Pathogenesis and prevention of cardiovascular disease in patients with chronic kidney disease

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Learn as if you were to live forever,
live as if you were to die tomorrow

Mohandas Gandhi

Why am I soft in the middle now
The rest of my life is so hard
I need a photo-opportunity
I want a shot at redemption
Don't want to end up a cartoon
In a cartoon graveyard

You can call me al: Paul Simon

Dedicated to my mother
as a mark of respect for her perseverance

Pathogenesis and prevention of cardiovascular disease in patients with chronic kidney disease

Contents

Chapter 1

General introduction and outline of the thesis

Cardiovascular disease in end-stage renal disease

Chronic kidney disease is a major health problem world-wide. In The United States, 106,912 patients developed end-stage renal disease (ESRD) during 2005 and there were 485,012 prevalent patients with ESRD in that year [1]. In The Netherlands, there were 13,000 patients with ESRD in 2005 of whom 45% were treated with chronic renal replacement therapy and 55% had received a renal transplant (www.reninet.nl).

As in the general population, cardiovascular disease is the major cause of death in patients with ESRD, accounting for about 40% of total mortality. However, life expectancy is severely reduced in ESRD patients compared to the general population, suggesting that the incidence and case-fatality of cardiovascular disease is increased in ESRD patients. Indeed, it has been shown that total and cardiovascular mortality is increased 20- to 30-fold in ESRD patients (Figure 1) [2;3]. The risk of non-fatal cardiovascular disease is also 10-30 times higher in patients with ESRD compared to the general population [4]. ESRD patients are therefore prone to the development and/or progression of cardiovascular disease.

Cardiovascular disease comprises a group of conditions, which can be divided in ischaemic and non-ischaemic conditions. Examples of non-ischaemic disease include cardiomyopathy, valvular heart disease, arrhythmia, pericarditis and pulmonary oedema. Ischaemic cardiovascular disease includes coronary artery disease, stroke, peripheral vascular disease and renovascular disease. Sudden death (cardiac arrest) is also frequently the result of ischaemic vascular disease. The typical arterial narrowing in ischaemic vascular disease is caused by atherosclerosis and thrombosis. Ischaemic forms of vascular disease account for the majority of cardiovascular cases in both the general and the ESRD population.

Several large randomised trials in ESRD patients have consistently shown no survival benefit from multiple new treatment strategies which were aimed to reduce cardiovascular disease, such as increased dialysis dose [5;6], homocysteine-lowering therapy [7], intensified nutrition [8], lipid-lowering with statins [9], treatment with angiotensin-converting enzyme inhibitors [10], and normalization of haemoglobin with erythropoetin [11;12]. While some of these interventions do have significant beneficial effects on the incidence of cardiovascular disease in the general population, the reason for the lack of benefit of these interventions in ESRD patients is unclear. One can postulate that the risk factors involved in the atherogenesis in patients with ESRD markedly differ from those of the general population or that the stage of atherosclerosis in these patients is so advanced that it has become resistant to the therapies that have been used.

Cardiovascular risk in mild to moderate kidney disease

Earlier stages of chronic kidney disease are more common than ESRD. Estimates suggest that 30 million Americans have some degree of chronic kidney disease (table 1) [13;14]. A survey conducted in the general population in Groningen, The Netherlands, demonstrated that prevalence of CKD, defined as a combination of estimated glomerular filtration rate (eGFR) < 60 ml/min or eGFR >60 ml/min + microalbuminuria / proteinuria, is 11% (table 2). Earlier stages of chronic kidney disease have also been associated with an increased cardiovascular morbidity and mortality (figure 2) [2;15-19] . In a recent review of 85 publications, involving a total of 552,258 subjects, it was concluded that there was an undeniable link between renal dysfunction and the development of cardiovascular disease [20]. The risk of cardiovascular disease (CVD) is already increased in early stages of chronic kidney

disease (at an estimated glomerular filtration rate (GFR) of around 75 ml/min) and increases continuously with decrease in renal function [20]. In addition, recent studies have also shown that patients with moderate chronic kidney disease are at a high risk of developing congestive heart failure [21] and that the majority of these patients have coronary heart disease [22;23]. A Norwegian study with 65,604 stage 2 to 3 CKD patients demonstrated that these subjects had a higher risk for development of premature cardiovascular death than progression to ESRD [24]. In addition, Keith *et al* also demonstrated that patients with stage 2, 3 and 4 CKD were more prone to develop CVD than ESRD[25]. Therefore, in our opinion, early stages of chronic kidney disease may primarily be considered as a cardiovascular problem.

Traditional risk factors

The clear association between slightly reduced kidney function and cardiovascular risk may, at least partly, be the result of a relationship between total atherosclerotic burden and decreased renal function, because intrarenal atherosclerosis (ischaemic renal disease) is a common cause of reduced renal function in patients with atherosclerosis [26]. However, even patients with a primary non-atherosclerotic renal disease such as polycystic kidney disease have an elevated risk of cardiovascular disease[27;28]. Traditional atherosclerotic risk factors such as age, dyslipidaemia, hypertension, diabetes mellitus, smoking and sedentary lifestyle play an important role in the occurrence of cardiovascular mortality in patients with chronic kidney disease [29]. However, these factors only partially explain the cardiovascular morbidity and mortality in patients with mild to advanced chronic kidney disease, suggesting a pathophysiological role for additional risk factors [30-33].

Stage	Description	GFR	Prevalence (N	%)
1	Kidney damage with normal or GFR >90	>90	5,900	3.3
2	Kidney damage with mild decrease in GFR	60 to 89	5,300	3.0
3	Moderate decrease in GFR	30 to 59	7,600	4.3
4	Severe decrease in GFR	15 to 29	400	0.2
5	Kidney failure	< 15 (or dialysis)	300	0.1

Note:

Glomerular filtration rate (GFR) was measured in ml/min/1.73m^2

Data for stages 1 to 4 from the Third National Health and Nutrition Examination Survey (1988 to 1994) include 177 million adults 20 years of age or older. Data for stage 5 from USRDS (1998) include approximately 230,000 patients treated by dialysis and assume 70,000 additional patients not on dialysis. GFR estimated from serum creatinine using MDRD study equation based on age, sex, race and calibration for serum creatinine. For stages 1 and 2 kidney damage was estimated by spot albumine to creatinine ratio of > 2.5 mg/µmol in men and > 3.5 mg/µmol in men on two measurements.

Table 1. Stages and prevalence of chronic kidney disease (adults 20 years of age or older) Source: K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Kidney Disease Outcome Quality Initiative. Am J Kidney Dis. 2002;39:S1-S246

eGFR mL/min	N	%	CKD stage	n	%
	8459			8459	
>90	2043	24 (23)	I (>90 + Alb)	225	2.7 (1.3)
60-90	5918	69 (71)	II (60-90 + Alb)	771	9.1 (3.8)
30-60	487	5.7 (5.3)	III (30-60)	487	5.7 (5.3)
15-30	8	0.1 (0.04)	IV (15-30)	8	0.1 (0.04)
<15	3	0.04 (0)	V (< 15)	3	0.04 (0)

eGFR is estimated with the simplified MDRD formula. CKD is staged according to the K/DOQI guidelines. CKD is defined as a GFR < 60 or a GFR > 60 + albuminuria > 30 mg/day (right panel). Because the total PREVEND study population of 8459 patients was enriched for the presence of microalbuminuria, distribution was also calculated in the true random sample from the general population (n = 2489). Percentages in parenthesis are given as those representing the distribution of eGFR and CKD in this unselected population cohort.

Abbreviations: eGFR, estimated glomerular filtration rate; MDRD, Modification of Diet in Renal Disease; PREVEND , Prevention of REnal and Vascular END-stage Disease; CKD, chronic kidney disease; K/DOQI, Kidney Disease Outcomes Quality Initiative; GFR, glomerular filtration rate; Alb, albuminuria.

Table 2. Distribution of renal function (eGFR) in the “general” population in Groningen, The Netherlands, in the PREVEND study

Adapted from: de Zeeuw D, Hillege HL, de Jong PE. The kidney, a cardiovascular risk marker, and a new target for therapy. *Kidney Int Suppl* 2005; S25-S29

Non-traditional risk factors

The so-called novel risk factors/markers (table 3) such as oxidative stress, endothelial dysfunction, protein-energy wasting, sympathetic overactivation and vascular calcification are highly prevalent and seem to play an important role in the development of atherosclerosis in patients with chronic kidney disease [31;34;35]. In this thesis, the role of several of these putative novel risk factors is further studied in patients with chronic kidney disease.

Non traditional (uraemia-related) risk factors
Oxidative stress
Fat mass: adipokine imbalance
Impaired one-carbon metabolism
Asymmetric dimethylarginine / endothelial dysfunction
Insulin resistance
Protein energy wasting
Anaemia
Vascular calcification
Symphathetic activation
Subclinical hypothyroidism
Uraemic bone disease
Volume overload
Coagulation disorders
Genetics/epigenetics

Table 3: novel cardiovascular risk factors in chronic kidney disease (adapted from Stenvinkel et al. Clin J Am Soc Nephrol 2008;3:505-521.
Risk factors which are being studied in this thesis are depicted in bold.

Oxidative stress:

Imbalance between production of reactive oxygen species (ROS) and anti-oxidant defence results in oxidative stress, which may arise either from deficiencies of anti-oxidants (such as glutathione, ascorbate or alpha-tocopherol) or increased formation of ROS [36]. Increased oxidative stress occurs in early stages of CKD and oxidation of low-density lipoproteins (LDL) is thought to be a key step in the initiation of atherosclerosis[37;38]. Therefore, the oxidative stress hypothesis may be considered as a unifying concept of increased CVD risk in CKD patients [34]. However, the causal relationship between oxidative stress and CVD in CKD patients is not clear and no intervention studies to examine the effects of oxidative stress reduction on CV events in CKD patients has been performed.

Adipokines:

Adipose tissue has a physiological role far beyond the mere storage of fat and recent interest has focused on the role of adipokines such as adiponectin [39] and leptin [40] both as protectors and promoters of CVD in patients with CKD. Adiponectin is secreted exclusively from adipocytes and is known to have anti-atherogenic and anti-inflammatory effects and also plays a role in glucose and lipid metabolism[41]. Leptin acts on a specific receptor located in the hypothalamus to decrease appetite and increase energy expenditure[42]. A few studies have shown that plasma levels of adiponectin and leptin are increased in patients with chronic renal failure[39;43;44]. Whether adiponectin and leptin play a pathophysiological role in endothelial dysfunction, inflammation, insulin resistance and dislipidaemia in patients with CKD is not clear, as published studies have shown contradicting results [45;46].

Impaired one-carbon metabolism:

In addition, impaired one-carbon metabolism has been recently recognized as one of the possible mechanisms responsible for increased atherogenicity in CKD patients [47;48]. In patients with ESRD, an elevated homocysteine concentration with impaired transsulphuration and remethylation of homocysteine, and elevated S-adenosylhomocysteine (SAH) levels, have been demonstrated [49;50]. Increased SAH inhibits methyltransferases, leading to impairment of methylation reactions. Global DNA hypomethylation has been demonstrated in dialysis patients and is implicated as an important candidate contributor to CVD in patients with ESRD [51;52]. Whether DNA hypomethylation is also a feature of earlier stages of renal insufficiency is currently unknown.

Asymmetric dimethylarginine (ADMA):

Asymmetric dimethyl arginine (ADMA) is an endogenous inhibitor of nitric oxide synthase and is derived from proteolysis of proteins containing methylated arginine residues. There is growing evidence that ADMA plays an important role in development of endothelial dysfunction and CVD in the general and in the ESRD population [53;54]. However, the role of ADMA in increased CVD in stage 2-4 CKD patients is unclear.

Surrogate markers of cardiovascular disease in patients with mild to moderate CKD:

Ideally, the identification and the effect of modification of (new) risk factors of atherosclerotic disease should be studied in large-scale prospective studies with relevant cardiovascular end-points. Over the last years, some valid markers of atherosclerotic disease have emerged, which can be used as surrogate end-points in studies on cardiovascular disease. These surrogate markers can roughly be divided in structural and functional parameters. The intima-media thickness of the common carotid artery is an example of a structural marker of vascular disease, and endothelial function parameters are used to provide functional data of the vascular system.

Intima-media thickness of the common carotid artery (CCA-IMT) is a strong surrogate marker of cardiovascular risk in the general [55] and ESRD [56] population. In addition, Desbien *et al*. recently demonstrated that decreased kidney function was strongly associated with a faster increase in CCA-IMT, which in turn was associated with more cardiovascular events [57]. Endothelial dysfunction, which can be characterized by increased plasma concentrations of endothelium-derived proteins such as von Willebrand factor (vWf), soluble vascular cell adhesion molecule-1 (sVCAM-1), or by reduced endothelium dependent brachial artery

flow mediated vasodilatation (BA-FMD), is strongly associated with many of the above-mentioned traditional and non-traditional cardiovascular risk factors [58]. Endothelial dysfunction has been demonstrated in patients with mild to moderate CKD and ESRD [59-61].

In conclusion, CCA-IMT and parameters of endothelial dysfunction such as BA-FMD, on the one hand, can be used to asses the eventual pathophysiological role of the traditional and non-traditional risk factors in the development of cardiovascular disease, and on the other hand to evaluate the effects of various treatment strategies, aimed at reducing the above-mentioned risk factors in patients with chronic kidney disease.

Although cardiovascular disease is highly prevalent in patients with mild to moderate kidney failure, only a few cardiovascular intervention studies have been done in these patients [62]. In addition, most of the large intervention trials with e.g. statins have excluded patients with moderate renal failure [62]. Given the magnitude of the problem, well-designed cross-sectional and intervention studies are needed to further elucidate the pathogenesis and to asses the effects of various treatment strategies on cardiovascular morbidity and mortality in patients with mild to moderate chronic kidney disease.

Outline of the thesis

In this thesis, a cohort of patient with mild to moderate kidney disease without manifest atherosclerotic disease was formed and it was examined whether:

1. kidney function was associated with non-traditional cardiovascular risk factors such as plasma adiponectin, plasma leptin, DNA-hypomethylation and plasma ADMA levels;

2. plasma ADMA levels and DNA-hypomethylation were associated with the common carotid artery intima-media thickness and endothelial function;

3. a stepwise, oxidative stress reducing treatment strategy with pravastatin, vitamin E and homocysteine-lowering treatment had any influence on CCA-IMT and BA-FMD.

Chapter 2 of this thesis reviews the complicated association between the two biochemically linked amino acids homocysteine and ADMA, and their pathophysiological role in the excess cardiovascular morbidity and mortality in patients with chronic kidney disease.
In chapter 3 we discuss the associations between renal function and plasma adiponectin and leptin levels. We also evaluate the possible role of these adipokines as protectors or promoters of cardiovascular disease in CKD.
In chapters 4 and 5 we describe the associations between renal function and plasma ADMA as well as DNA-hypomethylation. We further evaluate the association of these factors with the CCA-IMT in patients with CKD. In chapter 5, we also examine the effect of add-on treatment with pravastatin, vitamin E and homocysteine-lowering on DNA-hypomethylation.

In chapter 6, we examine the effect of the above-mentioned treatment strategy on CCA-IMT, brachial artery endothelial-dependent flow-mediated vasodilatation and urinary albumin level, all established surrogate markers of cardiovascular disease in patients with chronic kidney disease, and in chapter 7, the effect of this strategy on plasma ADMA levels.
The summary, main conclusions and future perspectives are provided in chapter 8.

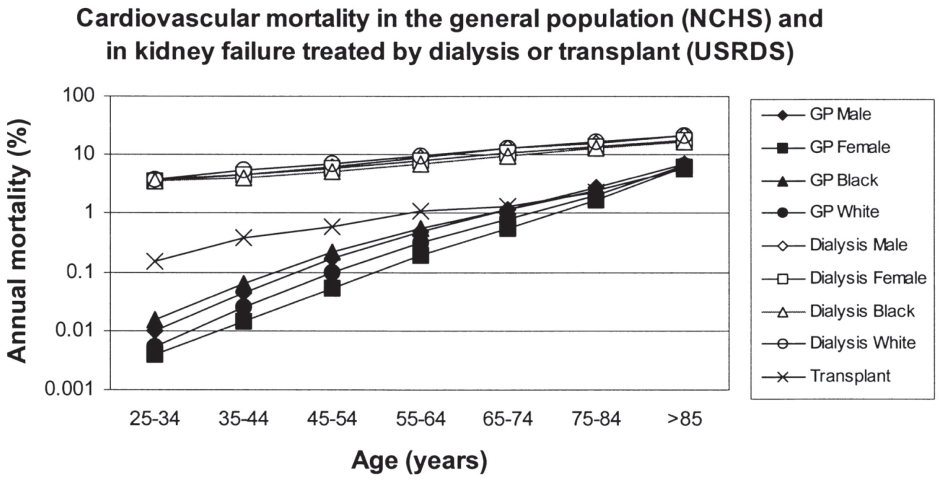

Figure 1. Cardiovascular mortality defined by death due to arrhythmias, cardiomyopathy, cardiac arrest, myocardial infarction, atherosclerotic heart disease, and pulmonary edema in general population (GP; National Center for Health Statistics [NCHS] multiple cause of mortality data files International Classification of Diseases, 9th Revision [ICD 9] codes 402, 404, 410 to 414, and 425 to 429, 1993) compared with kidney failure treated by dialysis or kidney transplant (United States Renal Data System [USRDS.]
Sarnak MJ et al. Hypertension 2003;42:1050-1065.

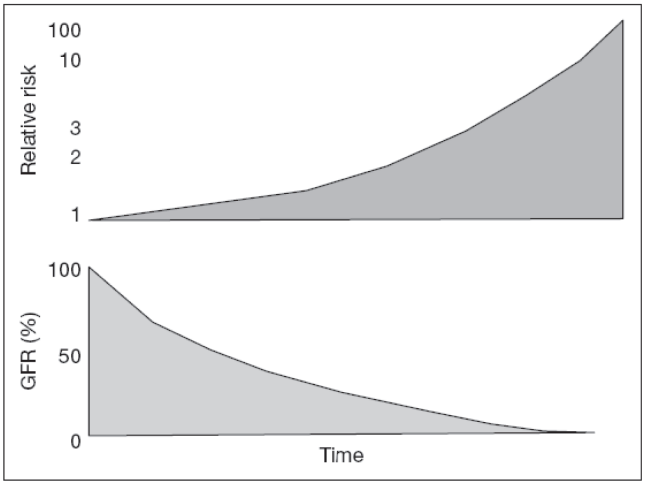

Figure 2. The relationship between estimated glomerular filtration rate and the risk of cardiovascular death

Zoccali C. The burden of cardiovascular disease in patients with chronic kidney disease and in end-stage renal disease. Contrib Nephrol. 2008;161:63-67.

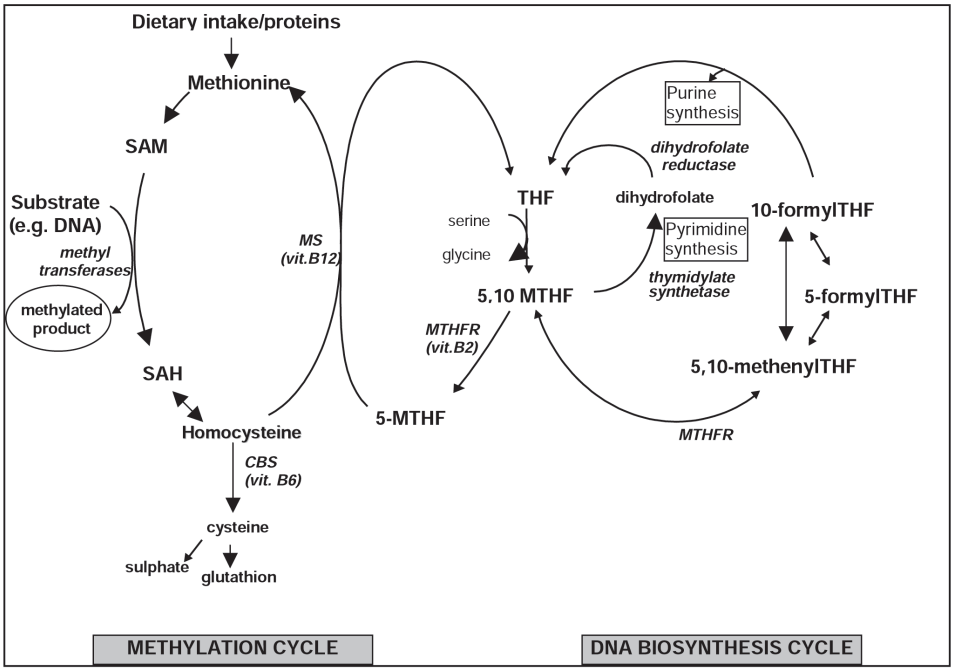

Figure 3: One-carbon metabolism

Methionine derived from the diet, protein breakdown or from remethylation of homocysteine forms S-adenosylmethionine (SAM or Ado Met). SAM can donate a methyl group by methyltransferases to the carbon 5' position of cytosine within the cytosine-guanine dinucleotide for DNA hypomethylation. The demethylated product of SAM, S-adenosylhomocysteine (SAH or Ado Hcy), is hydrolyzed further to homocysteine and adenosine (transmethylation). To prevent accumulation of homocysteine, it has to be hydrolyzed into cysteine by cysthationine-ß-synthase (CBS; transsulfuration) and leave the human body, or can be remethylated by a methyl-group of 5-methyltetrahydrofolate (5, MTHF) back into methionine.

(SAM; S-adenosylmethionine, SAH; S-adenosylhomocysteine, CBS; cystathionine ß-synthase, MTHF; methylenetetrahydrofolate, THF; tetrahydrofolate, MTHFR: 5,10 methylenetetrahydrofolate reductase, MS; methionine synthase).

Reference List

1. Collins AJ, Foley R, Herzog C *et al*. Excerpts from the United States Renal Data System 2007 annual data report. *Am J Kidney Dis* 2008; 51: S1-S320

2. Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. *J Am Soc Nephrol* 1998; 9: S16-S23

3. Foley RN, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998; 32: S112-S119

4. Levey AS, Beto JA, Coronado BE *et al*. Controlling the epidemic of cardiovascular disease in chronic renal disease: what do we know? What do we need to learn? Where do we go from here? National Kidney Foundation Task Force on Cardiovascular Disease. *Am J Kidney Dis* 1998; 32: 853-906

5. Eknoyan G, Beck GJ, Cheung AK *et al*. Effect of dialysis dose and membrane flux in maintenance hemodialysis. *N Engl J Med* 2002; 347: 2010-2019

6. Paniagua R, Amato D, Vonesh E *et al*. Effects of increased peritoneal clearances on mortality rates in peritoneal dialysis: ADEMEX, a prospective, randomized, controlled trial. *J Am Soc Nephrol* 2002; 13: 1307-1320

7. Jamison RL, Hartigan P, Kaufman JS *et al*. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. *JAMA* 2007; 298: 1163-1170

8. Cano NJ, Fouque D, Roth H *et al*. Intradialytic parenteral nutrition does not improve survival in malnourished hemodialysis patients: a 2-year multicenter, prospective, randomized study. *J Am Soc Nephrol* 2007; 18: 2583-2591

9. Wanner C, Krane V, Marz W *et al*. Atorvastatin in patients with type 2 diabetes mellitus undergoing hemodialysis. *N Engl J Med* 2005; 353: 238-248

10. Zannad F, Kessler M, Lehert P *et al*. Prevention of cardiovascular events in end-stage renal disease: results of a randomized trial of fosinopril and implications for future studies. *Kidney Int* 2006; 70: 1318-1324

11. Drueke TB, Locatelli F, Clyne N *et al*. Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N Engl J Med* 2006; 355: 2071-2084

12. Singh AK, Szczech L, Tang KL *et al*. Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med* 2006; 355: 2085-2098

13. Kopyt N. Management and treatment of chronic kidney disease. *Nurse Pract* 2007; 32: 14-23

14. Coresh J, Astor BC, Greene T, Eknoyan G, Levey AS. Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. *Am J Kidney Dis* 2003; 41: 1-12

15. Baigent C, Burbury K, Wheeler D. Premature cardiovascular disease in chronic renal failure. *Lancet* 2000; 356: 147-152

16. Drey N, Roderick P, Mullee M, Rogerson M. A population-based study of the incidence and outcomes of diagnosed chronic kidney disease. *Am J Kidney Dis* 2003; 42: 677-684

17. Henry RM, Kostense PJ, Bos G *et al*. Mild renal insufficiency is associated with increased cardiovascular mortality: The Hoorn Study. *Kidney Int* 2002; 62: 1402-1407

18. Manjunath G, Tighiouart H, Coresh J *et al*. Level of kidney function as a risk factor for cardiovascular outcomes in the elderly. *Kidney Int* 2003; 63: 1121-1129

19. Muntner P, He J, Hamm L, Loria C, Whelton PK. Renal insufficiency and subsequent death resulting from cardiovascular disease in the United States. *J Am Soc Nephrol* 2002; 13: 745-753

20. Vanholder R, Massy Z, Argiles A, Spasovski G, Verbeke F, Lameire N. Chronic kidney disease as cause of cardiovascular morbidity and mortality. *Nephrol Dial Transplant* 2005; 20: 1048-1056

21. Kottgen A, Russell SD, Loehr LR *et al*. Reduced kidney function as a risk factor for incident heart failure: the atherosclerosis risk in communities (ARIC) study. *J Am Soc Nephrol* 2007; 18: 1307-1315

22. Hyre AD, Fox CS, Astor BC, Cohen AJ, Muntner P. The impact of reclassifying moderate CKD as a coronary heart disease risk equivalent on the number of US adults recommended lipid-lowering treatment. *Am J Kidney Dis* 2007; 49: 37-45

23. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004; 351: 1296-1305

24. Hallan SI, Dahl K, Oien CM *et al*. Screening strategies for chronic kidney disease in the general population: follow-up of cross sectional health survey. *BMJ* 2006; 333: 1047

25. Keith DS, Nichols GA, Gullion CM, Brown JB, Smith DH. Longitudinal follow-up and outcomes among a population with chronic kidney disease in a large managed care organization. *Arch Intern Med* 2004; 164: 659-663

26. Tonelli M, Pfeffer MA. Kidney disease and cardiovascular risk. *Annu Rev Med* 2007; 58: 123-139

27. Fick GM, Johnson AM, Hammond WS, Gabow PA. Causes of death in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol* 1995; 5: 2048-2056

28. Handa SP. Cardiovascular manifestations of autosomal dominant polycystic kidney disease in young adults. *Clin Invest Med* 2006; 29: 339-346

29. Shlipak MG, Fried LF, Cushman M *et al*. Cardiovascular mortality risk in chronic kidney disease: comparison of traditional and novel risk factors. *JAMA* 2005; 293: 1737-1745

30. Muntner P, He J, Astor BC, Folsom AR, Coresh J. Traditional and nontraditional risk factors predict coronary heart disease in chronic kidney disease: results from the atherosclerosis risk in communities study. *J Am Soc Nephrol* 2005; 16: 529-538

31. Cheung AK, Sarnak MJ, Yan G *et al*. Atherosclerotic cardiovascular disease risks in chronic hemodialysis patients. *Kidney Int* 2000; 58: 353-362

32. Massy ZA, Taupin P, Jungers P, Landais P. Prediction model of coronary heart disease in patients with chronic kidney disease: role of plasma fibrinogen as a new prognostic variable. *Prilozi* 2005; 26: 63-77

33. Spiegel DM, Raggi P, Smits G, Block GA. Factors associated with mortality in patients new to haemodialysis. *Nephrol Dial Transplant* 2007; 22: 3568-3572

34. Himmelfarb J, Stenvinkel P, Ikizler TA, Hakim RM. The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney Int* 2002; 62: 1524-1538

35. Muntner P, Hamm LL, Kusek JW, Chen J, Whelton PK, He J. The prevalence of nontraditional risk factors for coronary heart disease in patients with chronic kidney disease. *Ann Intern Med* 2004; 140: 9-17

36. Halliwell B. The role of oxygen radicals in human disease, with particular reference to the vascular system. *Haemostasis* 1993; 23 Suppl 1: 118-126

37. Oberg BP, McMenamin E, Lucas FL *et al*. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 2004; 65: 1009-1016

38. Parthasarathy S, Santanam N, Auge N. Oxidized low-density lipoprotein, a two-faced Janus in coronary artery disease? *Biochem Pharmacol* 1998; 56: 279-284

39. Zoccali C, Mallamaci F, Tripepi G *et al*. Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease. *J Am Soc Nephrol* 2002; 13: 134-141

40. Scholze A, Rattensperger D, Zidek W, Tepel M. Low serum leptin predicts mortality in patients with chronic kidney disease stage 5. *Obesity (Silver Spring)* 2007; 15: 1617-1622

41. Hu E, Liang P, Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996; 271: 10697-10703

42. Mak RH, Cheung W, Cone RD, Marks DL. Leptin and inflammation-associated cachexia in chronic kidney disease. *Kidney Int* 2006; 69: 794-797

43. Becker B, Kronenberg F, Kielstein JT *et al*. Renal insulin resistance syndrome, adiponectin and cardiovascular events in patients with kidney disease: the mild and moderate kidney disease study. *J Am Soc Nephrol* 2005; 16: 1091-1098

44. Mak RH, Cheung W, Cone RD, Marks DL. Leptin and inflammation-associated cachexia in chronic kidney disease. *Kidney Int* 2006; 69: 794-797

45. Mitsnefes M, Kartal J, Khoury P, Daniels S. Adiponectin in children with chronic kidney disease: role of adiposity and kidney dysfunction. *Clin J Am Soc Nephrol* 2007; 2: 46-50

46. Guebre-Egziabher F, Drai J, Fouque D. Adiponectin and chronic kidney disease. *J Ren Nutr* 2007; 17: 9-12

47. Castro R, Rivera I, Blom HJ, Jakobs C, Tavares dA, I. Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview. *J Inherit Metab Dis* 2006; 29: 3-20

48. van Guldener C, Stam F, Stehouwer CD. Hyperhomocysteinaemia in chronic kidney disease: focus on transmethylation. *Clin Chem Lab Med* 2005; 43: 1026-1031

49. Loehrer FM, Angst CP, Brunner FP, Haefeli WE, Fowler B. Evidence for disturbed S-adenosylmethionine : S-adenosylhomocysteine ratio in patients with end-stage renal failure: a cause for disturbed methylation reactions? *Nephrol Dial Transplant* 1998; 13: 656-661

50. Mallamaci F, Zoccali C, Tripepi G *et al*. Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. *Kidney Int* 2002; 61: 609-614

51. Ingrosso D, Cimmino A, Perna AF *et al*. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. *Lancet* 2003; 361: 1693-1699

52. Perna AF, Capasso R, Lombardi C, Acanfora F, Satta E, Ingrosso D. Hyperhomocysteinemia and macromolecule modifications in uremic patients. *Clin Chem Lab Med* 2005; 43: 1032-1038

53. Furuki K, Adachi H, Matsuoka H *et al*. Plasma levels of asymmetric dimethylarginine (ADMA) are related to intima-media thickness of the carotid artery: an epidemiological study. *Atherosclerosis* 2007; 191: 206-210

54. Zoccali C, Bode-Boger S, Mallamaci F *et al*. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet* 2001; 358: 2113-2117

55. Hodis HN, Mack WJ, LaBree L *et al*. The role of carotid arterial intima-media thickness in predicting clinical coronary events. *Ann Intern Med* 1998; 128: 262-269

56. Benedetto FA, Mallamaci F, Tripepi G, Zoccali C. Prognostic value of ultrasonographic measurement of carotid intima media thickness in dialysis patients. *J Am Soc Nephrol* 2001; 12: 2458-2464

57. Desbien AM, Chonchol M, Gnahn H, Sander D. Kidney function and progression of carotid intima-media thickness in a community study. *Am J Kidney Dis* 2008; 51: 584-593

58. Zoccali C. The endothelium as a target in renal diseases. *J Nephrol* 2007; 20 Suppl 12: S39-S44

59. Tatematsu S, Wakino S, Kanda T *et al*. Role of nitric oxide-producing and -degrading pathways in coronary endothelial dysfunction in chronic kidney disease. *J Am Soc Nephrol* 2007; 18: 741-749

60. Stam F, van Guldener C, Schalkwijk CG, ter Wee PM, Donker AJ, Stehouwer CD. Impaired renal function is associated with markers of endothelial dysfunction and increased inflammatory activity. *Nephrol Dial Transplant* 2003; 18: 892-898

61. van Guldener C, Lambert J, Janssen MJ, Donker AJ, Stehouwer CD. Endothelium-dependent vasodilatation and distensibility of large arteries in chronic haemodialysis patients. *Nephrol Dial Transplant* 1997; 12 Suppl 2: 14-18

62. Tonelli M, Isles C, Curhan GC *et al*. Effect of pravastatin on cardiovascular events in people with chronic kidney disease. *Circulation* 2004; 110: 1557-1563

Chapter 2

Homocysteine and asymmetric dimethylarginine (ADMA): biochemically linked but differently related to vascular disease in chronic kidney disease.

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Abstract
Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, is formed by methylation of arginine residues in proteins and released after proteolysis. In this reaction, S-adenosylmethionine is methyldonor and S-adenosylhomocysteine the demethylated product. ADMA and homocysteine are thus biochemically linked. Both plasma homocysteine and ADMA concentrations are increased in patients with renal dysfunction, probably as a result an impairment in their metabolic, but not urinary, clearance. Hyperhomocysteinemia has been associated with an increased risk of cardiovascular disease in end-stage renal disease, especially in patients without malnutrition and inflammation. Also plasma ADMA levels have been associated with cardiovascular disease in renal failure patients. Both homocysteine and ADMA are thought to mediate their adverse vascular effects by impairing endothelial, nitric-oxide-dependent function resulting in decreased vasodilatation, increased smooth muscle cell proliferation, platelet dysfunction and increased monocyte adhesion. At the same time, it has been shown that the correlation between plasma ADMA and homocysteine is weak and that, in renal patients, the association of plasma ADMA carotid intima-media thickness, cardiovascular events and overall mortality is independent of homocysteine. This indicates that the negative vascular effects of ADMA and homocysteine have a different etiology. Treatment with folic acid treatment substantially lowers homocysteine, but not ADMA concentration. So far, homocysteine-lowering therapy has not been very successful in decreasing cardiovascular disease. In patients with renal failure, ADMA reduction may be an interesting new goal in the prevention of cardiovascular disease.

Introduction
Cardiovascular disease is the leading cause of death both in the general population as in patients with end-stage renal disease (ESRD). However, mortality rates from cardiovascular disease are 10 to 30 times higher in ESRD than in the general population (1;2), suggesting that atherosclerosis is accelerated in ESRD. More recently, data have shown that patients with milder forms of chronic kidney disease (CKD) with an estimated GFR of 15-60 ml/min, also have an elevated risk of mortality and cardiovascular disease (3;4). The excess of mortality and cardiovascular events in ESRD and CKD patients is independent of traditional risk factors for atherosclerotic disease, such as hypertension, diabetes, smoking and hyperlipidemia (1-4). Alternative risk factors that may contribute to cardiovascular disease in renal failure include hyperhomocysteinemia and elevated plasma levels of asymmetric dimethylarginine (ADMA).

Homocysteine
Homocysteine is a sulfhydryl amino acid, which is formed by demethylation of methionine. S-adenosylmethionine is the intermediate in this reaction and serves as a universal methyl donor. Homocysteine is either remethylated to methionine or transsulfurated to cysteine. In plasma, about 80 percent of homocysteine is protein-bound. When it was found that patients with extreme hyperhomocysteinemia due to different genetic enzyme defects suffered from premature atherosclerosis and venous thrombosis, it was hypothesized that homocysteine was a direct vasculotoxic agent (5). Subsequently, it was shown that plasma homocysteine is strongly related to renal function and that virtually all ESRD patients have elevated homocysteine levels, probably due to decreased metabolic clearance (6). Pro-

spective studies examining the relationship between hyperhomocysteinemia and cardiovascular events in renal patients, have shown equivocal results (7;8). The most recent data indicate that hyperhomocysteinemia is indeed a risk marker for cardiovascular disease in ESRD, but that malnutrition/inflammation can confound this association (9;10).

Asymmetric dimethylarginine (ADMA)

ADMA is a naturally occurring amino acid in plasma and in cells that is formed by methylation of arginine residues in proteins and released when these proteins are hydrolysed. Other products of arginine methylation are L-monomethyl-L-arginine (L-NMMA) and symmetric dimethylarginine (SDMA). ADMA and L-NMMA, but not SDMA, are inhibitors of nitric oxide (NO) synthase, the enzyme that facilitates the conversion from arginine to NO and citrulline. Because its plasma level is 10 times higher than that of L-NMMA, ADMA has gained much interest as a potential regulator of the arginine-NO pathway, and thereby of vascular disease.

ADMA production is facilitated by the enzyme protein arginine methyltransferase 1 (PRMT1) which methylates arginine residues in histones and nuclear RNA-binding proteins. ADMA is eliminated by breakdown to citrulline and methylamine (catalysed by dimethylarginine dimethylaminohydrolase, DDAH) and to a lesser extent by urinary excretion.

Like hyperhomocysteinemia, elevated plasma ADMA concentrations have first been described in patients with renal failure (11). Later, milder elevations of plasma ADMA levels have also been found in patients with peripheral arterial disease (12), hypertension (13), hypercholesterolemia (14), type 2 diabetes (15) and (postmethionine loading) hyperhomocysteinemia (16;17).

Potential mechanisms of elevated plasma ADMA levels in renal failure are increased protein methylation, increased proteolysis, impaired renal excretion and impaired metabolism by DDAH. Protein methylation by PRMT may be increased in renal failure due to enhanced shear stress (18) or increased PRMT gene expression (19). However, stable isotope studies with labeled methionine have indicated that total body transmethylation is rather decreased in ESRD (20;21). Protein degradation is a complex process, but there are indications that overall proteolysis is not enhanced in ESRD (22). Impaired renal excretion contributes to elevated plasma ADMA levels, but the relationship between renal function and plasma level is stronger for SDMA than for ADMA (23). The major factor for high plasma ADMA levels in renal failure seems to be decreased DDAH activity, which in turn may be due to increased oxidative stress and/or hyperhomocysteinemia.

An elevated plasma ADMA level has been identified as a cardiovascular risk factor in the general (24;25) and in the ESRD population (26;27).

Relationship between homocysteine and ADMA

Biochemically, homocysteine and ADMA are linked in several ways (Figure 1). First, methylation of arginine to L-NMMA and from L-NMMA to ADMA yields two molecules of homocysteine. Second, homocysteine may enhance protein degradation by destabilizing protein structure or by increasing oxidative stress, resulting in ADMA release. Third, homocysteine inhibits DDAH, the enzyme responsible for the breakdown of ADMA (28).

Homocysteine and ADMA share many of the presumed pathophysiological mechanisms that link these compounds to vascular disease. Most of these mechanisms are related to impaired NO-dependent endothelial function, leading to vasoconstriction, hypertension,

platelet activation, proliferation of smooth-muscle cells and monocyte adhesion. Mechanisms that are more specific for homocysteine include increased oxidative stress, protein homocysteinylation/acylation, endoplasmic reticulum stress and hypomethylation, although the latter may theoretically also be caused by elevated ADMA levels. More ADMA-specific vascular effects comprise left ventricular hypertrophy, reduced sodium excretion and inhibition of angiogenesis.

On the basis of the biochemical links, one would expect a firm relationship between plasma homocysteine and ADMA levels. Somewhat surprisingly, studies in siblings of patients with vascular disease and postmethionine hyperhomocysteinemia (29), type 2 diabetes with hyperhomocysteinemia (29), patients at high vascular risk (30) and patients with peripheral vascular disease (31) have shown that this relationship is non-significant or marginal at most. Also in patients with renal failure, which is thought to be a common causal factor for the elevation of both plasma levels, the correlation between plasma homocysteine and ADMA was non-significant in two studies of 225 (26) and 197 hemodialysis patients (27) and in one study of 93 CKD patients (32). These observations suggest that other factors, probably involved in the elimination of homocysteine and ADMA, are more important predictors of their plasma levels.

Homocysteine, ADMA and relationship with vascular disease in renal failure

As outlined earlier, both homocysteine and ADMA are predictors of cardiovascular events in ESRD patients (9;10;26;27). Interestingly, in the two studies in which both homocysteine and ADMA were analyzed, it was found that higher ADMA, but not homocysteine levels were associated with cardiovascular disease (26;27). One other study suggested that low ADMA levels were associated with cardiovascular events in renal disease, but the studied population was rather small and heterogeneous (33).

The relationship of homocysteine and ADMA with vascular disease in renal patients has also been studied using measurement of the carotid intima-media thickness (IMT), an accepted surrogate marker of atherosclerotic disease (34). In ESRD patients, carotid IMT is increased and predicts cardiovascular mortality (35-38). Most studies in renal patients have found no significant relationship between homocysteine and carotid IMT (27;32;39-41). In one study, homocysteine was associated with carotid IMT in hemodialysis patients at baseline, but not with the change of carotid IMT over time (42). The latter finding was confirmed in another study (40). The relationship between plasma ADMA level and carotid IMT may be more consistent (27;32;42). Studies that have included both ADMA and homocysteine in the analyses show that only ADMA is a predictor of the carotid IMT in renal patients (32;42).

It can be concluded that plasma ADMA level is a stronger predictor of cardiovascular events and carotid IMT in renal patients than plasma homocysteine.

Homocysteine-lowering treatment

Folic acid based regimens lower plasma homocysteine level in renal patients by 20-50%, depending on the baseline vitamin supplementation. So far, however, homocysteine-lowering treatment has not been able to clearly improve endothelial function (43), prevent progression of carotid IMT (44), or reduce cardiovascular events (44;45) in these patients. Lowering of homocysteine can be expected to lower plasma ADMA by protein stabilization and by improving DDAH activity. However, controlled studies in subjects without renal dys-

function show that homocysteine-lowering does not substantially influence plasma ADMA (29). The effect of homocysteine-lowering treatment on plasma ADMA levels in ESRD is unknown. One small study in renal transplant recipients suggests that folic acid acid does not decrease ADMA (46).

ADMA lowering?

Lowering of plasma ADMA could be an important new tool in cardiovascular risk prevention in renal failure patients. So far, however, there is no specific ADMA-lowering therapy. Limited success has been reported of interventions with inhibitors of the renin-angiotensin-aldosterone system, statins, fibrates, rosiglitazone, metformin, estrogen replacement therapy, fish oil and anti-oxidants (47).

In the ATIC trial, 93 patients with CKD were randomized to an oxidative-stress-reducing treatment consisting of pravastatin with addition of vitamin E after 6 months and homocysteine-lowering B-vitamins after 12 monts, or to placebo (48). Active treatment was associated with a reduction of carotid IMT after 18 months. Baseline ADMA level correlated with carotid IMT and at present further analyses are being done to examine whether ADMA levels were reduced by the treatment and/or related to the observed beneficial vascular effects.

Conclusion

Patients with renal failure exhibit an excess of cardiovascular events, that is not completely explained by traditional risk factors. Homocysteine and ADMA are amino acids, which are biochemically linked by a common synthetic pathway. Plasma levels of homocysteine and ADMA are elevated in patients with renal failure and both have been associated with cardiovascular events, possibly due to their negative effects on endothelial function. Despite these similarities, plasma homocysteine and ADMA are not strongly related and studies that have examined both compounds, have shown that only ADMA predicts cardiovascular events. Also in contrast to homocysteine, ADMA is related to carotid IMT, an important surrogate vascular risk marker. Homocysteine-lowering treatment does not lower ADMA and has not been shown to reduce cardiovascular events and mortality in ESRD patients. Specific ADMA-reduction is a new and promising target to beneficially influence cardiovascular disease in renal failure patients.

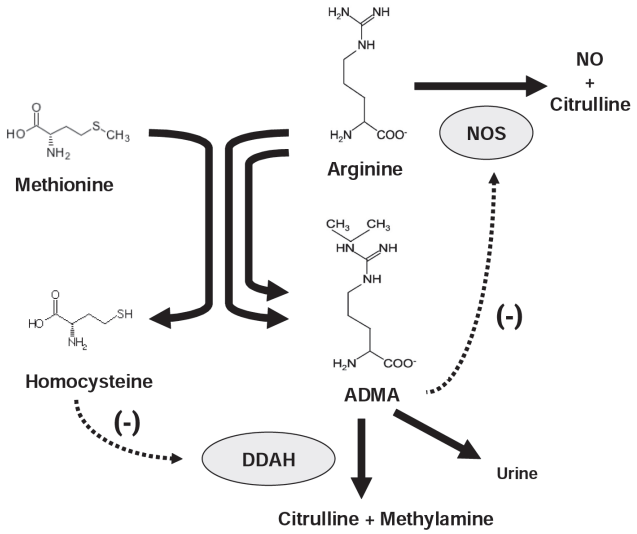

Figure 1. Metabolic link between homocysteine and asymmetric dimethylarginine.
Two methyl groups from methionine are used for posttransscriptional methylation of arginine, yielding homocysteine and ADMA. ADMA inhibits the conversion of arginine to nitric oxide and citrulline. ADMA can be excreted in the urine, but is mainly degraded to citrulline and methylamine, a process that can be inhibited by homocysteine.
NO: nitric oxide, NOS: nitric oxide synthase, ADMA: asymmetric dimethylarginine, DDAH: dimethylarginine dimethylaminohydrolase.

Reference List

(1) Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. J Am Soc Nephrol. 1998;9:S16-S23.

(2) Herzog CA, Ma JZ, Collins AJ. Poor long-term survival after acute myocardial infarction among patients on long-term dialysis. N Engl J Med. 1998;339:799-805.

(3) Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med. 2004;351:1296-305.

(4) Stam F, van Guldener C, Becker A, Dekker JM, Heine RJ, Bouter LM et al. Endothelial dysfunction contributes to renal function-associated cardiovascular mortality in a population with mild renal insufficiency: the Hoorn study. J Am Soc Nephrol. 2006;17:537-45.

(5) McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol. 1969;56:111-28.

(6) van Guldener C. Why is homocysteine elevated in renal failure and what can be expected from homocysteine-lowering? Nephrol Dial Transplant. 2006;21:1161-66.

(7) Mallamaci F, Zoccali C, Tripepi G, Fermo I, Benedetto FA, Cataliotti A et al. Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. Kidney Int. 2002;61:609-14.

(8) Kalantar-Zadeh K, Block G, Humphreys MH, McAllister CJ, Kopple JD. A low, rather than a high, total plasma homocysteine is an indicator of poor outcome in hemodialysis patients. J Am Soc Nephrol. 2004;15:442-53.

(9) Ducloux D, Klein A, Kazory A, Devillard N, Chalopin JM. Impact of malnutrition-inflammation on the association between homocysteine and mortality. Kidney Int. 2006;69:331-35.

(10) Suliman M, Stenvinkel P, Qureshi AR, Kalantar-Zadeh K, Barany P, Heimburger O et al. The reverse epidemiology of plasma total homocysteine as a mortality risk factor is related to the impact of wasting and inflammation. Nephrol Dial Transplant. 2007;22:209-17.

(11) Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 1992;339:572-75.

(12) Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation. 1997;95:2068-74.

(13) Surdacki A, Nowicki M, Sandmann J, Tsikas D, Boeger RH, Bode-Boeger SM et al. Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension. J Cardiovasc Pharmacol. 1999;33:652-58.

(14) Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O et al. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation. 1998;98:1842-47.

(15) Stuhlinger MC, Abbasi F, Chu JW, Lamendola C, McLaughlin TL, Cooke JP et al. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. JAMA. 2002;287:1420-26.

(16) Holven KB, Haugstad TS, Holm T, Aukrust P, Ose L, Nenseter MS. Folic acid treatment reduces elevated plasma levels of asymmetric dimethylarginine in hyperhomocysteinaemic subjects. Br J Nutr. 2003;89:359-63.

(17) Wanby P, Brattstrom L, Brudin L, Hultberg B, Teerlink T. Asymmetric dimethylarginine and total homocysteine in plasma after oral methionine loading. Scand J Clin Lab Invest. 2003;63:347-53.

(18) Osanai T, Saitoh M, Sasaki S, Tomita H, Matsunaga T, Okumura K. Effect of shear stress on asymmetric dimethylarginine release from vascular endothelial cells. Hypertension. 2003;42:985-90.

(19) Matsuguma K, Ueda S, Yamagishi S, Matsumoto Y, Kaneyuki U, Shibata R et al. Molecular mechanism for elevation of asymmetric dimethylarginine and its role for hypertension in chronic kidney disease. J Am Soc Nephrol. 2006;17:2176-83.

(20) Stam F, van Guldener C, ter Wee PM, Kulik W, Smith DE, Jakobs C et al. Homocysteine clearance and methylation flux rates in health and end-stage renal disease: association with S-adenosylhomocysteine. Am J Physiol Renal Physiol. 2004;287:F215-F223.

(21) van Guldener C, Stam F, Stehouwer CD. Hyperhomocysteinaemia in chronic kidney disease: focus on transmethylation. Clin Chem Lab Med. 2005;43:1026-31.

(22) Raj DS, Oladipo A, Lim VS. Amino Acid and protein kinetics in renal failure: an integrated approach. Semin Nephrol. 2006;26:158-66.

(23) Kielstein JT, Salpeter SR, Bode-Boeger SM, Cooke JP, Fliser D. Symmetric dimethylarginine (SDMA) as endogenous marker of renal function—a meta-analysis. Nephrol Dial Transplant. 2006;21:2446-51.

(24) Valkonen VP, Paiva H, Salonen JT, Lakka TA, Lehtimaki T, Laakso J et al. Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine. Lancet. 2001;358:2127-28.

(25) Boger RH, Maas R, Schulze F, Schwedhelm E. Elevated levels of asymmetric dimethylarginine (ADMA) as a marker of cardiovascular disease and mortality. Clin Chem Lab Med. 2005;43:1124-29.

(26) Zoccali C, Bode-Boger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L et al. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet. 2001;358:2113-17.

(27) Kumagai H, Sakurai M, Takita T, Maruyama Y, Uno S, Ikegaya N et al. Association of homocysteine and asymmetric dimethylarginine with atherosclerosis and cardiovascular events in maintenance hemodialysis patients. Am J Kidney Dis. 2006;48:797-805.

(28) Stuhlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP. Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. Circulation. 2001;104:2569-75.

(29) Spoelstra-de Man AM, Teerlink T, Brouwer CB, Rauwerda JA, Stehouwer CD, Smulders YM. No effect of B vitamins on ADMA levels in patients at increased cardiovascular risk. Clin Endocrinol (Oxf). 2006;64:495-501.

(30) Schmitt B, Wolters M, Kressel G, Hulsmann O, Strohle A, Kuhn-Velten WN et al. Effects of combined supplementation with B vitamins and antioxidants on plasma levels of asymmetric dimethylarginine (ADMA) in subjects with elevated risk for cardiovascular disease. Atherosclerosis. 2007;193:168-76.

(31) Ziegler S, Mittermayer F, Plank C, Minar E, Wolzt M, Schernthaner GH. Homocyst(e)ine-lowering therapy does not affect plasma asymmetrical dimethylarginine concentrations in patients with peripheral artery disease. J Clin Endocrinol Metab. 2005;90:2175-78.

(32) Nanayakkara PW, Teerlink T, Stehouwer CD, Allajar D, Spijkerman A, Schalkwijk C et al. Plasma asymmetric dimethylarginine (ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration in patients with mild-to-moderate renal failure. Kidney Int. 2005;68:2230-36.

(33) Busch M, Fleck C, Wolf G, Stein G. Asymmetrical (ADMA) and symmetrical dimethylarginine (SDMA) as potential risk factors for cardiovascular and renal outcome in chronic kidney disease - possible candidates for paradoxical epidemiology? Amino Acids. 2006;30:225-32.

(34) Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. Circulation. 2007;115:459-67.

(35) Benedetto FA, Mallamaci F, Tripepi G, Zoccali C. Prognostic value of ultrasonographic measurement of carotid intima media thickness in dialysis patients. J Am Soc Nephrol. 2001;12:2458-64.

(36) Kato A, Takita T, Maruyama Y, Kumagai H, Hishida A. Impact of carotid atherosclerosis on long-term mortality in chronic hemodialysis patients. Kidney Int. 2003;64:1472-79.

(37) Nishizawa Y, Shoji T, Maekawa K, Nagasue K, Okuno S, Kim M et al. Intima-media thickness of carotid artery predicts cardiovascular mortality in hemodialysis patients. Am J Kidney Dis. 2003;41:S76-S79.

(38) Kawagishi T, Nishizawa Y, Konishi T, Kawasaki K, Emoto M, Shoji T et al. High-resolution B-mode ultrasonography in evaluation of atherosclerosis in uremia. Kidney Int. 1995;48:820-26.

(39) Leskinen Y, Lehtimaki T, Loimaala A, Huhtala H, Salenius JP, Oja SS et al. Homocysteine and carotid atherosclerosis in chronic renal failure—the confounding effect of renal function. Atherosclerosis. 2004;175:315-23.

(40) Suliman ME, Stenvinkel P, Jogestrand T, Maruyama Y, Qureshi AR, Barany P et al. Plasma pentosidine and total homocysteine levels in relation to change in common carotid intima-media area in the first year of dialysis therapy. Clin Nephrol. 2006;66:418-25.

(41) Zoungas S, Branley P, Kerr PG, Ristevski S, Muske C, Demos L et al. Atherosclerosis and folic acid supplementation trial in chronic renal failure: baseline results. Nephrology (Carlton). 2004;9:130-41.

(42) Zoccali C, Benedetto FA, Maas R, Mallamaci F, Tripepi G, Malatino LS et al. Asymmetric dimethylarginine, C-reactive protein, and carotid intima-media thickness in end-stage renal disease. J Am Soc Nephrol. 2002;13:490-96.

(43) van Guldener C, Janssen MJ, Lambert J, ter Wee PM, Jakobs C, Donker AJ et al. No change in impaired endothelial function after long-term folic acid therapy of hyperhomocysteinaemia in haemodialysis patients. Nephrol Dial Transplant. 1998;13:106-12.

(44) Zoungas S, McGrath BP, Branley P, Kerr PG, Muske C, Wolfe R et al. Cardiovascular morbidity and mortality in the Atherosclerosis and Folic Acid Supplementation Trial (ASFAST) in chronic renal failure: a multicenter, randomized, controlled trial. J Am Coll Cardiol. 2006;47:1108-16.

(45) Wrone EM, Hornberger JM, Zehnder JL, McCann LM, Coplon NS, Fortmann SP. Randomized trial of folic acid for prevention of cardiovascular events in end-stage renal disease. J Am Soc Nephrol. 2004;15:420-26.

(46) Austen SK, Fassett RG, Geraghty DP, Coombes JS. Folate supplementation fails to affect vascular function and carotid artery intima media thickness in cyclosporin A-treated renal transplant recipients. Clin Nephrol. 2006;66:373-79.

(47) Beltowski J, Kedra A. Asymmetric dimethylarginine (ADMA) as a target for pharmacotherapy. Pharmacol Rep. 2006;58:159-78.

(48) Nanayakkara PW, van Guldener C, ter Wee PM, Scheffer PG, van Ittersum FJ, Twisk JW et al. Effect of a treatment strategy consisting of pravastatin, vitamin E, and homocysteine lowering on carotid intima-media thickness, endothelial function, and renal function in patients with mild to moderate chronic kidney disease: results from the Anti-Oxidant Therapy in Chronic Renal Insufficiency (ATIC) Study. Arch Intern Med. 2007;167:1262-70.

Chapter 3

Plasma adiponectin concentration has an inverse and a non linear association with estimated glomerular filtration rate in patients with K/DOQI 3-5 chronic kidney disease

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Submitted

Abstract:

Background
Chronic kidney disease (CKD) is associated with an increased incidence of cardiovascular disease (CVD). A few studies have demonstrated elevated plasma adiponectin and leptin levels in CKD. The aims of this study were to assess whether 1) the estimated glomerular filtration rate (eGFR) is associated with plasma leptin and adiponectin; and 2) adiponectin and leptin (partly) explain associations of CKD with endothelial dysfunction, insulin resistance, and low-grade inflammation in patients with K/DOQI stage 3-5 CKD.

Methods
Baseline data from 91 patients with stage 3-4 CKD in the Anti-oxidant Therapy in Chronic renal insufficiency study, a randomized, double-blind, placebo-controlled trial in which the effects of oxidative stress-lowering treatment on vascular function and structure were studied, and from 50 dialysis naïve patients who took part in an open-label, randomised study that compared two peritoneal dialysis regimens were used in the analysis. All subjects for both the studies were recruited in the same centres.

Results
The association between eGFR and adiponectin was non-linear. In multivariate analysis, log-eGFR (unstandardized ß = ¯8.303 μg/ml, $P < 0.0001$) was the strongest determinant of adiponectin and body mass index the strongest determinant of leptin (ß = 2.477 ng/ml, $P < 0.0001$). Plasma adiponectin and leptin did not modify the associations between eGFR and plasma von Willebrand factor or soluble vascular adhesion molecule-1. Plasma leptin had the strongest association with the homeostatic model assessment (HOMA-IR) index. Plasma C-reactive protein had no association with adiponectin or leptin.

Conclusions
In patients with K/DOQI stage 3-5 CKD, renal function had a significant non-linear inverse association and was the strongest predictor of adiponectin. BMI was the strongest predictor of plasma leptin. Plasma adiponectin and leptin did not explain, and thus presumably are not involved in, the association between eGFR and markers of endothelial dysfunction.

Introduction
Patients with chronic kidney disease (CKD) have an increased risk of cardiovascular disease which is not fully explained by the presence of known cardiovascular risk factors.[1;2] Therefore, other atherogenic mechanisms are thought to be active in CKD patients. Adipose tissue is increasingly being recognized as an endocrine organ involved in regulation of energy homeostasis and metabolism.[3] Several hormones (adipokines) such as adiponectin and leptin are secreted from adipose tissues. Adiponectin is secreted exclusively from adipocytes and is known to have anti-atherogenic and anti-inflammatory effects.[4;5] Adiponectin also plays a role in glucose and lipid metabolism.[6] Low circulating levels of adiponectin are associated with endothelial dysfunction and are often found in populations at risk of CVD.[7] For example, plasma adiponectin is decreased in patients with obesity-linked disorders, [8] coronary artery disease and dyslipidaemia.[9] These findings suggest that higher adiponectin levels confer a protective effect against atherosclerosis. Somewhat

unexpectedly, a few studies have shown that plasma levels of adiponectin are elevated in diabetic and non-diabetic patients with end-stage renal disease (ESRD) and moderately severe chronic kidney disease (CKD).[10-12]

Leptin acts on a specific receptor located in the hypothalamus to decrease appetite and increase energy expenditure.[13] However, in humans leptin has also been found to be positively associated with insulin resistance, diabetes risk, C-reactive protein (CRP) and blood pressure, and is inversely associated with high-density lipoprotein cholesterol levels.[14] In addition, leptin concentration also increases with obesity and correlates strongly with percentage body fat, probably due to a state of "leptin resistance" in these patients.[15] Plasma levels of leptin are also increased in chronic renal failure, probably because of decreased renal clearance.[13]

Whether adiponectin and leptin play a pathophysiological role in endothelial dysfunction[16], inflammation[16], insulin resistance[10], and dislipidaemia[17] in patients with CKD is not clear, as published studies have shown contradicting results For example, while Guebre-Egziabher *et al* reported that the increase in adiponectin in patients with CKD is explained primarily by patients body composition and the altered metabolic parameters [18], Mitsnefes *et al* atribute this increase primarly to the decline in kidney function.[19] While one study reports that lower adiponectin level is associated with increased risk of cardiovascular events in CKD patients[11] others report that, high rather than low, adiponectin levels predicts mortality in both CKD [20] and congestive heart failure.[21] In addition, functional role of increased leptin in CKD patients is also unclear.[22]

In view of these considerations, we investigated, in individuals with a wide range of estimated glomerular filtration rates (K/DOQI stages 3-5), whether renal function was associated with plasma adiponectin and leptin levels. In addition, we investigated whether adiponectin or leptin (partly) explained the known associations between renal function and a marker of endothelial dysfunction {plasma von Willebrand factor (vWf)} and a marker of leukocyte-endothelial cell adhesion {soluble leukocyte endothelial cell adhesion molecule-1 (sVCAM-1)}, inflammation (CRP) and insulin resistance (HOMA model).

Methods

To examine the influence of a wide range of estimated glomerular filtration rate (eGFR) on plasma adiponectin and leptin we decided to combine databases of two studies which were being performed in our institute. The first study, the Anti-oxidant Therapy In Chronic renal insufficiency (ATIC) study is a randomised, double-blind, placebo-controlled trial in which the effects of oxidative stress-lowering treatment on vascular function and structure are studied in patients with chronic non-diabetic renal failure (estimated glomerular filtration rate (eGFR) of 15-60 mL / min per 1.73m^2 according to the modification of Diet in Renal Disease (MDRD) study equation (Levey equation 7) [23;24] who are free from manifest arterial occlusive disease. Participants in the trial were randomised to active treatment consisting of add-on therapy with pravastatin, vitamin E and homocysteine-lowering therapy, or to placebo. Subjects not using angiotensin converting enzyme inhibitors (ACE-inhibitors) or angiotensin receptor blockers (ARBs) at inclusion were put on ACE-inhibitors for at least two weeks before the baseline measurement and randomisation. Those who were on ARBs continued their ARBs. Individuals with diabetes mellitus (ADA criteria), active vasculitis, nephrotic syndrome (>3g/24h urine protein), renal transplantation, fasting total cholesterol

> 7 mmol/L, cholesterol-lowering therapy within three months prior to inclusion or known ischaemic cardiac, cerebrovascular or peripheral arterial disease were excluded. Baseline data of the ATIC study was used for this analysis.

In addition, we included baseline data of patients starting with an open-label, randomized, multi-centre study that compared two peritoneal dialysis regimens (NEPP studie).[25] All patients were 18 years or older, in stable condition with an estimated life expectancy of more than one year. Only incident CAPD patients who had not previously undergone renal replacement therapy were included in the study. In contrast to the ATIC study, patients with diabetes mellitus and known ischaemic cardiac, cerebrovascular or peripheral arterial disease were included in the NEPP study. All the patients who took part in the ATIC and NEPP study were recruited from seven outpatients nephrology and internal medical clinics in Amsterdam, the Netherlands. Baseline data from 91 patients with stage 3-5 CKD (mean eGFR ± SD: 32 ± 13 mL / min per 1.73m^2 range: 11 – 60 mL / min per 1.73m^2) who took part in the ATIC-study and 50 patients with stage 5 CKD (mean eGFR ± SD: 7 ± 3 mL / min per 1.73m^2, range: 2 – 15 mL / min per 1.73m^2) who took part in the NEPP-study were used in this evaluation. ACE-inhibitors were used by 75% and 50% of patients in the ATIC and NEPP study respectively. Patients in the ATIC study were statin naïve and less than 20% patients in the NEPP study used statins. Non of the patients used homocysteine lowering therapy or other vitamins. Written informed consent was obtained from all participants.

Procedures

All patients were examined in a fasting state and a detailed history was obtained including medication use and clinical manifestations of peripheral, cerebral and coronary vascular disease. Height and weight were measured with the individuals wearing light clothing. Blood samples were collected following an overnight fasting period and after 15 minutes of rest in supine position. Samples were immediately placed on ice and centrifuged within 15 minutes. Plasma was stored at –80 °C until analysis.

Laboratory analyses

Plasma total adiponectin was measured using sandwich immunoassay (R & D Systems, Minneapolis, USA), with intra- and inter-assay coefficients of variation of 5 and 7% respectively. Plasma leptin was measured in duplicate by radioimmunoassay (Mediagnost Gmbh, Tübingen, Germany) using standards and a ^{125}I-tracer prepared from recombinant leptin and a highly polyclonal antibody that specifically and quantitatively recognise human leptin with an intra- and inter-assay coefficients of variation of 3 and 5% respectively.
Total cholesterol, HDL cholesterol and triglycerides were measured by routine laboratory methods. We calculated LDL cholesterol by use of Friedewald formula. Serum creatinine concentration was assessed by a kinetic Jaffé method, with an intra- and inter-assay coefficients of variation of 1.4 and 2.2% respectively. Plasma concentrations of CRP were measured with a highly sensitive in-house ELISA with rabbit anti-CRP (Dako, Copenhagen, Denmark) as a capturing and tagging antibody, with intra- and inter-assay coefficients of variation of 3.8 and 4.7%, respectively.

Plasma vWf antigen was measured by an enzyme-linked immunosorbent assay (ELISA), with rabbit anti-vWf antigen as a capturing antibody and a peroxidase-conjugated rabbit

anti-vWf antigen as a detecting antibody (Dako, Copenhagen, Denmark). The concentration of vWf was expressed as a percentage of the antigen concentration in normal pooled plasma, which is defined as 100%. The intra- and interassay variations were 2.3 and 3.8%, respectively. sVCAM-1 was measured by an ELISA method (Diaclone, Besancon, France) with an intra- and interassay coefficient of variation of 4.0 and 8.0% respectively. Insulin resistance was estimated by using the homeostatic model assessment (HOMA-IR) index [plasma glucose level X plasma insulin level /22.5], which has been validated with the euglycaemic-hyperinsulinaemic clamp method in subjects with various degrees of glucose tolerance [26] and in patients with chronic kidney disease [27].

Renal function was estimated by the MDRD study equation (estimated GFR in ml/min/1.73 m^2) = 170 x [plasma creatinine µmol/L x 0.0113] $^{-0.999}$ x [age] $^{-0.176}$ x [plasma urea mmol/L x 2.8] $^{-0.170}$ x [Albumine g/L x 0.1] $^{+0.318}$ x [0.762 if female] x [1.18 if black][24].

Statistical analyses

All analyses were carried out with the SPSS 11.5 software program (SPSS, Chicago, IL, USA). Linear regression analysis was performed to investigate the associations between renal function on the one hand and adiponectin, leptin, vWf, s-VCAM-1, CRP and HOMA-IR index on the other hand. In addition, linear regression analysis was performed to investigate the associations between leptin and adiponectin on the one hand and vWf, sVCAM-1, CRP and HOMA-IR index on the other hand. All associations were first analysed without adjustments and then with adjustments for factors known to be important in the physiology of the given parameter (for example during the analysis of adiponectin and leptin we adjusted for age, body mass index (BMI), sex and proteinuria), and variables that were found to be significantly associated with a given parameter in the univariate analyses. During the regression analyses residual plots were produced after each analysis and parameters were log transformed if the residuals were not normally distributed. P-values < 0.05 were considered statistically significant.

Results

Table 1 shows baseline characteristics of the participants. The underlying causes of renal disease in these patients were hypertension (50 patients), polycystic kidney disease (13 patients), obstructive uropathy (16 patients), glomerulonephritis (11 patients), drug-induced (9 patients), vesico-urethral reflux (9 patients), diabetes mellitus (9 patients), IgA nephropathy (2 patients), undetermined (11 patients), miscellaneous (11 patients).

Renal function and plasma total adiponectin

The inverse association between estimated glomerular filtration rate (eGFR) and adiponectin (Figure 1) became linear after log transformation of eGFR (log-eGFR). Therefore, we used log-eGFR as an independent variable in the linear regression analyses. In univariate analysis there was a significant inverse association between log-eGFR and plasma adiponectin (unstandardized ß for log-MDRD = ¯ 8.303 µg/ml, P < 0.0001).

In addition, we also found significant inverse associations between BMI and adiponectin (unstandardized ß for BMI = ¯ 0.693 µg/ml, P = 0.001), sex and adiponectin (higher in females: ß = ¯ 4.451 µg/ml, P = 0.02), and significant direct associations between HDL-cholesterol and adiponectin (ß for HDL-cholesterol = 8.599 µg/ml, P < 0.0001), urinary

protein and adiponectin (ß for urinary protein = 1.392 μg/ml, P = 0.03).
After mutual adjustment, only log-eGFR (unstandardized ß = ¯8.661 μg/ml, *P* < 0.0001) and HDL-cholesterol (ß = 6.172 μg/ml, *P* = 0.001) remained significant determinants of plasma adiponectin.

Renal function and plasma leptin

In univariate analysis there was a significant linear association between eGFR and plasma leptin (unstandardized ß for eGFR = ¯ 0.348 ng/ml, *P* = 0.004). In addition, there were significant univariate associations between BMI and leptin (ß for BMI = 2 ng/ml, P < 0.0001), sex and leptin (higher in females: ß = ¯ 17 ng/ml, P < 0.0001), and urinary protein and leptin (ß = 4 ng/ml, P = 0.001). There were no significant associations between age, HDL-cholesterol and leptin.

After mutual adjustment, BMI (unstandardized ß = 2.477 ng/ml, *P*< 0.0001), sex (higher in females ß = 16.740 ng/ml, *P*< 0.0001), eGFR (ß = ¯0.342 ng/ml, *P* = 0.001) and urinary protein (ß = 2.354 ng/ml, *P* = 0.02) remained significant determinants of plasma leptin.

Renal function, adiponectin, leptin and markers of endothelial dysfunction

In the univariate analysis there was a significant inverse associations between eGFR and plasma vWf (ß = ¯ 1.79%, P < 0.0001). Adjustment for adiponectin (ß for association between eGFR and plasma vWf =¯ 1.55 %, P < 0.0001) and leptin (ß for association between eGFR and plasma vWf =¯ 1.67%, P < 0.0001) did not change the significant association between eGFR and vWf.

In the univariate analysis there were significant inverse associations between eGFR and plasma sVCAM-1 (ß = ¯ 5.814 ng/ml, P < 0.0001). Adjustment for adiponectin (ß for association between eGFR and plasma vWf = ¯5.706 ng/ml, P < 0.0001) and leptin (ß for association between eGFR and plasma vWf =¯ 5.658 ng/ml, P < 0.0001) did not change the significant association between eGFR and sVCAM-1.

Renal function, adipokines and HOMA- IR index

There was a significant direct association between plasma leptin and HOMA-IR index (unstandardized ß = 0.055, P < 0.0001), which was not affected by adjustment for BMI, eGFR, plasma vWf, plasma sVCAM, plasma CRP and age.

Plasma CRP, AGE, BMI and adiponectin had no significant association with HOMA-IR index. Exclusion of 15 patients with diabetes had no influence on these results.

In the univariate analysis there was a significant inverse associations between eGFR and HOMA – IR index (ß = ¯ 0.212 ng/ml, P = 0.01) which was not affected by adjustment for BMI.

Renal function and plasma CRP

There were no significant associations between plasma CRP on the one hand and renal function, serum adiponectin, serum leptin, HOMA-IR index, sex, or BMI on the other.

Discussion

In this population with K/DOQI stage 3-5 CKD patients we demonstrate that eGFR has the strongest inverse association with plasma adiponectin in univariate and multivariate analysis. Furthermore, eGFR had a significant positive association with serum leptin. However, BMI was the strongest predictor of plasma leptin during univariate and multivariate analysis. Plasma adiponectin and leptin had no influence on the significant association between eGFR and markers of endothelial dysfunction (plasma vWf and plasma sVCAM-1). Plasma leptin was also associated strongly with HOMA-IR index in univariate and multivariate analyses.

Renal function had the strongest association with plasma adiponectin in our population. A few authors have also reported that adiponectin levels are increased in patients with ESRD [28] and in mild to moderate chronic kidney disease [29]. Whether this increase reflects impaired adiponectin clearance by the kidney or whether this increase in adipokines has a functional role in counteracting increased cardiovascular risk known to associated with CKD is not elucidated yet. Guebre-Egziabher *et al.* reported that in 48 patients with chronic kidney disease (eGFR: mean ± SD; 53.5 ± 24.9, range; 12 -107 ml/min/1.73m^2) adiponectin had a linear and inverse significant association with eGFR, urinary albumin excretion and BMI. In their multivariate analysis, BMI was a stronger predictor of plasma adiponectin levels in comparison to eGFR [18]. They concluded that adiponectin in chronic kidney disease is related more to metabolic disturbances than to decline in renal function. However, the 141 patients in our study had a lower eGFR and a narrower eGFR range (mean ± SD; 24 ± 16, range 4 – 60 ml/min/1.73m^2) compared to their study and we showed for the first time that the relationship between eGFR and serum adiponectin was nonlinear. In addition eGFR had the strongest association with plasma adiponectin during the univariate and multivariate analyses.

We also demonstrated that, in our population, adiponectin was positively associated with plasma vWf and plasma sVCAM-1, established markers of endothelial dysfunction and leukocyte-endothelial cell adhesion respectively.[30] However, these significant associations disappeared after addition of eGFR to the model (data not shown). In addition, adiponectin had no influence on the significant associations between eGFR on the one hand and plasma vWf and sVCAM-1 on the other. We therefore conclude that increased adiponectin in CKD patients is primarily a reflection of impaired kidney function and plasma adiponectin does not explain the known associations between kidney function and endothelial dysfunction. Our results are also in line with a recent study which demonstrated that renal transplantation is accompanied by a significant reduction of plasma adiponectin suggesting that impaired adiponectin elimination or biodegradation may be responsible for the elevation of plasma adiponectin [31].

As demonstrated in few other studies we also showed a direct and a significant association between plasma adiponectin and HDL-cholesterol in univariate and multivariate analysis [32-34]. Rothenbacher *et al* demonstrated that adiponectin was strongly associated with HDL-cholesterol in patients with coronary heart disease [35]. This association was also shown in a population with normal weight [36] and in obese subjects [37]. Mechanisms responsible for this association are unknown. However, in a previous study it has been suggested that

adiponectin stimulates peroxisome proliferator-activated receptor α (PPAR-α) activity in both skeletal muscles and liver thereby stimulating the synthesis of HDL-cholesterol.

Plasma leptin is cleared from the circulation by the kidney through glomerular filtration followed by metabolic degradation in the renal tubules.[38;39] As expected leptin had a strong direct association with eGFR in the univariate and multivariate analysis in our population. However, BMI had the strongest association with plasma leptin in univariate and multivaraiate analysis (mean BMI in our population 26±5 kg/m^2). In addition, during the univariate and multivariaate analysis leptin had the strongest direct association with HOMA-IR index, an established marker of insulin resistance.

In the general population, leptin inhibits appetite and increases energy expenditure. However, many obese individuals have inappropriately high levels of circulating leptin which is attributed to hyporesponsiveness to leptin in obesity [40]. A few studies show a paradoxically inverse association between higher plasma leptin and improved markers of nutritional status in patients with CKD [41]. Two recent studies in dialysis patients demonstrated a strong direct association between plasma leptin and both BMI (r = 0.70) and triceps skin fold thickness (r = 0.77).[42;43] A higher leptin level was also associated with higher serum lipids, healthier clinical characteristics, a lower atherosclerosis score and a better appetite. Recent study, that followed 71 haemodialysis patients during 7 years also showed that lower baseline leptin level was associated with higher mortality (3.8 -times higher risk of death among the patients with a serum leptin concentration below median).[44] This phenomenon may be another example of “reverse epidemiology” [45] similar to the “obesity paradox” [46] or “hypercholesterolaemia paradox” [47] in CKD patients as low BMI is associated with increased mortality in CKD patients.[48] In our population we also showed a direct and significant association between leptin and insulin resistance and BMI, both markers of improved nutritional status. Whether this is the result of leptin resistance has not yet been elucidated and future studies are needed to clarify the role leptin in CKD patients. We thus conclude that, although renal function strongly predicted plasma leptin in our population BMI and HOMA-IR index also had a strong association with plasma leptin in univariate and multivariate analysis. However, plasma leptin did not explain the known associations between kidney function and endothelial dysfunction.

Study limitations

The cross-sectional nature of the study does not permit any final conclusions with regard to the causality of described associations. Because we aimed to analyse a population of patients with a wide range of eGFR we decided to pool the databases of two studies which were performed in our institute. This may certainly have introduced some bias. However, all the patients for both studies were recruited in the same centres and ATIC study included patients with mild to moderate renal failure and the patients in the NEPP study were patients who were being prepared to start peritoneal dialysis. The average age of the patients in ATIC study was 54 ± 12 and NEPP study 57 ± 15 years. Although, in our opinion, two populations were similar in their characteristics and these studies were not designed to study any new mechanisms which explains the link between kidney failure, adiponectin and leptin. There were 15 patients with diabetes mellitus and all these patients had an eGFR = 21 ml/min/1.73m^2. Exclusion of these patients did not change any of the given results.

We determined total adiponectin in our patients. However, adiponectin circulates in low, middle and high molecular weight (HMW) isomers and clinical data suggest that the high molecular isoform is the most important correlate of insulin sensitivity.[49] It has been suggested that in patients with renal failure low levels of HMW isoform may be masked by an overall increase in low and middle molecular weight isomers which may explain susceptibility of these patients to insulin resistance and vascular disease.[50] However Shen *et al* recently demonstrated that in patients with kidney disease the paradox of high adiponectin is not explained by decreased levels of HMW adiponectin.[50] Thus determination total and not HMW adiponectin probably did not have a significant influence on the outcomes in our study.

In conclusion, we demonstrated for the first time that plasma adiponectin had a significant, non-linear, inverse association with eGFR and in multivariate analysis eGFR had the strongest correlation with plasma adiponectin. Plasma adiponectin had no influence on the significant association between eGFR and markers of endothelial dysfunction, i.e. plasma vWf and plasma sVCAM-1. We therefore conclude that increased adiponectin in CKD patients is primarily a reflection of impaired kidney function. Furthermore, plasma leptin also had a significant positive association with eGFR. However, markers of nutritional status such as BMI and HOMA-IR index were strongly associated plasma leptin during univariate and multivariate analysis. Future studies are needed to clarify the role leptin in CKD patients.

N = 141 eGFR = 2 - 60 (23 ± 16) ml/min/1.73m^2	N = 48 eGFR = 2 – 11 (7 ± 2) ml/min/1.73m^2	N = 46 eGFR = 12 – 30 (21 ± 6) ml/min/1.73m^2	N = 47 eGFR = 31 – 60 (43 ± 8) ml/min/1.73m^2
Male gender N (%)	26 (54%)	28 (61%)	27 (57%)
Age years	56 ± 15	55 ± 13	54 ± 11
Body mass index kg/m^2	25 ± 4	25 ± 4	27 ± 5
Blood pressure mmHg			
Systolic	157 ± 27	140 ± 23	134 ± 21
Diastolic	91 ± 14	80 ± 12	78 ± 12
Lipids *mmol/L*			
Total cholesterol	4.9 ± 1.1	5.5 ± 1.2	5.7 ± 1.1
LDL cholesterol	3.2 ± 1.1	3.4 ± 1.0	3.7 ± 0.8
HDL cholesterol	1.3 ± 0.5	1.3 ± 0.4	1.2 ± 0.3
Triglycerides	1.8 ±1.0	1.9 ± 1.1	1.9 ± 1.0
Plasma adiponectin median (range) µg/ml			
Total population	18.70 (3.32 – 75.07)	4.74 (0.72 – 16.13)	3.57 (0.90 – 25.64)
In females	19.24 (4.22 – 75.07)	9.13 (3.54 – 16.13)	5.20 (1.77 – 25.64)
In males	17.15 (3.31 – 42.69)	3.50 (0.72 – 15.16)	2.79 (0.90 – 11.39)
Plasma leptin median (range) ng/ml			
Total population	24.46 (0.27 – 100.49)	7.48 (0.01 – 77.73)	9.77 (0.36 – 58.13)
In females	24.46 (1.39 – 100.49)	19.65 (2.40 – 77.73)	30.27 (1.30 – 58.13)
In males	28.30 (0.27 – 75.50)	3.29 (0.01 – 28.16)	4.90 (0.36 – 34.78)
History of cardiovascular disease n(%)	8 (16%)	4 (9%)	0 (0%)
Plasma von Willebrand factor (%)	200 ± 85	169 ± 53	139 ± 49
Plasma sVCAM-1	1200 ± 287	1052 ± 254	956 ± 222
Plasma CRP median (range) mg/l	3.07 (0.20-45.28)	3.83 (0.15 – 24..98)	2.29 (0.12 – 26.11)
Urinary protein (g/day)	1.58 (0.21 – 8.80)	0.93 (0.09 – 6.21)	0.36 (0.06 – 3.46)
Diabetes n(%)	12 (25%)	3 (6%)	0 (0%)
ACE-inhibitors / AT Receptor blockers n(%)	24 (50%)	38 (82%)	39 (83%)

Table 1: baseline characteristics of the participants divided into tertiles according to estimated glomerular filtration rate (eGFR) measured with modification of diet in renal disease (MDRD) formula

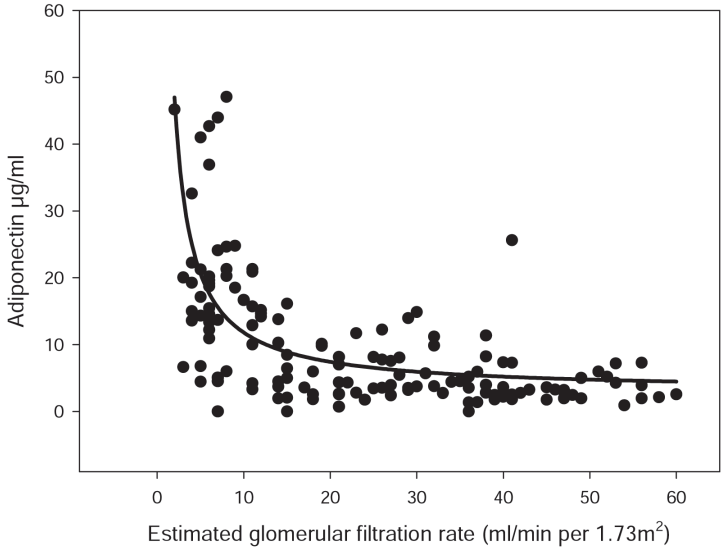

Figure 1: The association between plasma adiponectin and estimated glomerular filtration rate according to the modification of Diet in Renal Disease (MDRD) study equation (Levey equation 7)

Reference List

1. Foley RN, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998; 32: S112-S119

2. Henry RMA, Kostense PJ, Bos G *et al*. Mild renal insufficiency is associated with increased cardiovascular mortality: The Hoorn Study. *Kidney Int* 2002; 62: 1402-1407

3. Halvorsen YD, Wilkison WO, Briggs MR. Human adipocyte proteomics—a complementary way of looking at fat. *Pharmacogenomics* 2000; 1: 179-185

4. Ouchi N, Kihara S, Arita Y *et al*. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 1999; 100: 2473-2476

5. Ouchi N, Kihara S, Arita Y *et al*. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 2000; 102: 1296-1301

6. Hu E, Liang P, Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996; 271: 10697-10703

7. Tan KCB, Xu A, Chow WS *et al*. Hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation. *J Clin Endocrinol Metab* 2004; 89: 765-769

8. Arita Y, Kihara S, Ouchi N *et al*. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999; 257: 79-83

9. Weyer C, Funahashi T, Tanaka S *et al*. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 2001; 86: 1930-1935

10. Becker B, Kronenberg F, Kielstein JT *et al*. Renal insulin resistance syndrome, adiponectin and cardiovascular events in patients with kidney disease: the mild and moderate kidney disease study. *J Am Soc Nephrol* 2005; 16: 1091-1098

11. Zoccali C, Mallamaci F, Tripepi G *et al*. Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease. *J Am Soc Nephrol* 2002; 13: 134-141

12. Guebre-Egziabher F, Drai J, Fouque D. Adiponectin and chronic kidney disease. *J Ren Nutr* 2007; 17: 9-12

13. Mak RH, Cheung W, Cone RD, Marks DL. Leptin and inflammation-associated cachexia in chronic kidney disease. *Kidney Int* 2006; 69: 794-797

14. Lawlor DA, Smith GD, Kelly A, Sattar N, Ebrahim S. Leptin and coronary heart disease risk: prospective case control study of British women. *Obesity (Silver Spring)* 2007; 15: 1694-1701

15. Mohamed-Ali V, Pinkney JH, Coppack SW. Adipose tissue as an endocrine and paracrine organ. *Int J Obes Relat Metab Disord* 1998; 22: 1145-1158

16. Stam F, van Guldener C, Schalkwijk CG, ter Wee PM, Donker AJM, Stehouwer CDA. Impaired renal function is associated with markers of endothelial dysfunction and increased inflammatory activity. *Nephrol Dial Transplant* 2003; 18: 892-898

17. Molitch ME. Management of dyslipidemias in patients with diabetes and chronic kidney disease. *Clin J Am Soc Nephrol* 2006; 1: 1090-1099

18. Guebre-Egziabher F, Bernhard J, Funahashi T, Hadj-Aissa A, Fouque D. Adiponectin in chronic kidney disease is related more to metabolic disturbances than to decline in renal function. *Nephrol Dial Transplant* 2005; 20: 129-134

19. Mitsnefes M, Kartal J, Khoury P, Daniels S. Adiponectin in children with chronic kidney disease: role of adiposity and kidney dysfunction. *Clin J Am Soc Nephrol* 2007; 2: 46-50

20. Menon V, Li L, Wang X *et al*. Adiponectin and mortality in patients with chronic kidney disease. *J Am Soc Nephrol* 2006; 17: 2599-2606

21. Kistorp C, Faber J, Galatius S *et al*. Plasma adiponectin, body mass index, and mortality in patients with chronic heart failure. *Circulation* 2005; 112: 1756-1762

22. Axelsson J, Stenvinkel P. Role of fat mass and adipokines in chronic kidney disease. *Curr Opin Nephrol Hypertens* 2008; 17: 25-31

23. Nanayakkara PWB, Teerlink T, Stehouwer CDA *et al*. Plasma asymmetric dimethylarginine (ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration in patients with mild-to-moderate renal failure. *Kidney Int* 2005; 68: 2230-2236

24. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999; 130: 461-470

25. le Poole CY, Welten AGA, Weijmer MC, Valentijn RM, van Ittersum FJ, ter Wee PM. Initiating CAPD with a regimen low in glucose and glucose degradation products, with icodextrin and amino acids (NEPP) is safe and efficacious. *Perit Dial Int* 2005; 25 Suppl 3: S64-S68

26. Bonora E, Targher G, Alberiche M *et al*. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 2000; 23: 57-63

27. Shoji T, Emoto M, Nishizawa Y. HOMA index to assess insulin resistance in renal failure patients. *Nephron* 2001; 89: 348-349

28. Huang JW, Yen CJ, Chiang HW, Hung KY, Tsai TJ, Wu KD. Adiponectin in peritoneal dialysis patients: a comparison with hemodialysis patients and subjects with normal renal function. *Am J Kidney Dis* 2004; 43: 1047-1055

29. Schalkwijk CG, Chaturvedi N, Schram MT, Fuller JH, Stehouwer CDA. Adiponectin is inversely associated with renal function in type 1 diabetic patients. *J Clin Endocrinol Metab* 2006; 91: 129-135

30. Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. *J Thromb Haemost* 2006; 4: 1186-1193

31. Chudek J, Adamczak M, Karkoszka H *et al*. Plasma adiponectin concentration before and after successful kidney transplantation. *Transplant Proc* 2003; 35: 2186-2189

32. Cnop M, Havel PJ, Utzschneider KM *et al*. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. *Diabetologia* 2003; 46: 459-469

33. Schulze MB, Rimm EB, Shai I, Rifai N, Hu FB. Relationship between adiponectin and glycemic control, blood lipids, and inflammatory markers in men with type 2 diabetes. *Diabetes Care* 2004; 27: 1680-1687

34. Tschritter O, Fritsche A, Thamer C *et al*. Plasma adiponectin concentrations predict insulin sensitivity of both glucose and lipid metabolism. *Diabetes* 2003; 52: 239-243

35. Rothenbacher D, Brenner H, Marz W, Koenig W. Adiponectin, risk of coronary heart disease and correlations with cardiovascular risk markers. *Eur Heart J* 2005; 26: 1640-1646

36. Yamamoto Y, Hirose H, Saito I *et al*. Correlation of the adipocyte-derived protein adiponectin with insulin resistance index and serum high-density lipoprotein-cholesterol, independent of body mass index, in the Japanese population. *Clin Sci (Lond)* 2002; 103: 137-142

37. Yang WS, Lee WJ, Funahashi T *et al*. Plasma adiponectin levels in overweight and obese Asians. *Obes Res* 2002; 10: 1104-1110

38. Cumin F, Baum HP, de Gasparo M, Levens N. Removal of endogenous leptin from the circulation by the kidney. *Int J Obes Relat Metab Disord* 1997; 21: 495-504

39. Cumin F, Baum HP, Levens N. Mechanism of leptin removal from the circulation by the kidney. *J Endocrinol* 1997; 155: 577-585

40. Considine RV, Sinha MK, Heiman ML *et al*. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996; 334: 292-295

41. Don BR, Rosales LM, Levine NW, Mitch W, Kaysen GA. Leptin is a negative acute phase protein in chronic hemodialysis patients. *Kidney Int* 2001; 59: 1114-1120

42. Aguilera A, Bajo MA, Rebollo F *et al*. Leptin as a marker of nutrition and cardiovascular risk in peritoneal dialysis patients. *Adv Perit Dial* 2002; 18: 212-217

43. Iglesias P, Diez JJ, Fernandez-Reyes MJ *et al*. Effects of short-term recombinant human growth hormone therapy on plasma leptin concentrations in dialysis patients. *Nephrol Dial Transplant* 2002; 17: 260-264

44. Scholze A, Rattensperger D, Zidek W, Tepel M. Low serum leptin predicts mortality in patients with chronic kidney disease stage 5. *Obesity (Silver Spring)* 2007; 15: 1617-1622

45. Kalantar-Zadeh K, Block G, Humphreys MH, Kopple JD. Reverse epidemiology of cardiovascular risk factors in maintenance dialysis patients. *Kidney Int* 2003; 63: 793-808

46. Kalantar-Zadeh K, Abbott KC, Salahudeen AK, Kilpatrick RD, Horwich TB. Survival advantages of obesity in dialysis patients. *Am J Clin Nutr* 2005; 81: 543-554

47. Kilpatrick RD, McAllister CJ, Kovesdy CP, Derose SF, Kopple JD, Kalantar-Zadeh K. Association between serum lipids and survival in hemodialysis patients and impact of race. *J Am Soc Nephrol* 2007; 18: 293-303

48. Kovesdy CP, Anderson JE, Kalantar-Zadeh K. Paradoxical association between body mass index and mortality in men with CKD not yet on dialysis. *Am J Kidney Dis* 2007; 49: 581-591

49. Pajvani UB, Hawkins M, Combs TP *et al*. Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem* 2004; 279: 12152-12162

50. Shen YY, Charlesworth JA, Kelly JJ, Loi KW, Peake PW. Up-regulation of adiponectin, its isoforms and receptors in end-stage kidney disease. *Nephrol Dial Transplant* 2007; 22: 171-178

Chapter 4

Plasma asymmetric dimethylarginine ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration in patients with mild to moderate renal failure

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Abstract

Background

Patients with renal insufficiency have an increased risk of cardiovascular disease, which is not fully explained by the presence of known cardiovascular risk factors. In patients with end-stage renal disease, increased serum concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), has been linked to excess cardiovascular morbidity. We investigated, in patients with mild to moderate renal failure, the relationship between plasma ADMA and three surrogate markers of atherosclerosis that have been shown to have prognostic value, namely carotid intima-media thickness (IMT), plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) and plasma C-reactive protein (CRP).

Methods

We used baseline data of an ongoing randomised trial in which the effects of oxidative stress-lowering treatment on vascular function and structure are studied in patients with chronic non-diabetic renal failure without clinical evidence of atherosclerosis (GFR 15 to 70 ml / min / per 1.73m^2 according to the Cockcroft-Gault equation; ATIC study).

Results

Data from ninety-three patients were used. Creatinine clearance was inversely related to plasma ADMA concentration (standardized β after adjustment = -0.342, P = 0.023). Plasma ADMA was strongly related to carotid IMT in univariate (β = 0.459, P<0.0001) and multivariate analysis (β = 0.444, P<0.0001). Plasma ADMA was also significantly related with plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) in univariate (β = 0.260, P = 0.010) and multivariate (β = 0.242, P = 0.022) analysis. Plasma ADMA was not significantly related to C-reactive protein (β = -0.134, P = 0.204).

Conclusions

In patients with mild to moderate renal failure, renal function is inversely associated with plasma ADMA, which, in turn, is positively associated with carotid IMT and plasma sVCAM-1 concentration. Increased plasma ADMA may be a link between renal function and cardiovascular disease in patients with mild to moderate renal failure.

Introduction

Patients with end-stage renal disease have an increased risk of cardiovascular disease [1]. A similar association exists between mild to moderate renal insufficiency and cardiovascular events [2,3]. This association is not fully explained by the presence of known cardiovascular risk factors such as hypertension, diabetes, smoking, homocysteine level and dyslipidemia [2,4]. Other atherogenic mechanisms thus seem to be active in patients with renal insufficiency.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthase and is derived from the proteolysis of proteins containing methylated arginine residues. It has considerable biological effects, particularly in the cardiovascular system [5,6,7]. When administered acutely, ADMA inhibits NO synthase catalytic activity, endothelium-dependent NO-mediated vascular responses and endothelium-dependent NO bioavailability, all of

which are reversed by L-arginine [5]. The plasma concentrations of ADMA and its biologically inactive stereoisomer -symmetric dimethylarginine (SDMA) are two to six times higher in patients with end-stage renal failure than in healthy controls [5,8]. Plasma ADMA concentrations in dialysis patients are strongly and independently related to the intima-media thickness (IMT) of the carotid artery [9]. Carotid IMT is a strong surrogate marker of cardiovascular risk in the general [10] and dialysis [11] population. In addition, plasma ADMA concentrations are higher in dialysis patients with clinically manifest atherosclerosis than in those without atherosclerosis [12]. Furthermore, ADMA levels were predictive of future cardiovascular events and overall mortality in a cohort of patients undergoing hemodialysis [13].

Data on the relationship between ADMA and cardiovascular disease in patients with mild to moderate renal failure (GFR between 10 to 70 ml/min) have not been reported. In order to gain more insight into the mechanism of atherosclerosis in mild to moderate renal failure, we therefore examined the relationship between plasma ADMA and carotid IMT, using baseline data of the Anti-oxidant Therapy In Chronic renal insufficiency (ATIC) study. In addition, we studied the relationship between plasma ADMA on the one hand and plasma C-reactive protein (CRP) and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) on the other. CRP and sVCAM-1 are markers of inflammatory activity and leukocyte-endothelial cell adhesion, respectively, and have been shown to be markers of cardiovascular risk [14,15,16,17].

Methods

Patients

Between May 2001 and December 2002, individuals with a creatinine clearance of 15-70 mL / min per 1.73m^2 (according to the Cockcroft-Gault equation) from seven (six nephrology and one internal medicine) out-patients clinics in Amsterdam, The Netherlands, were screened for eligibility for participation in the Anti-oxidant Therapy In Chronic renal insufficiency (ATIC) study. The ATIC study is a randomised, double- blind, placebo-controlled trial in which the effects of oxidative stress-lowering treatment on vascular function and structure are studied in patients with chronic non-diabetic renal failure who are free from manifest arterial occlusive disease. Participants in the trial were randomised to active treatment consisting of add-on therapy with pravastatin, vitamin E and homocysteine-lowering therapy, or to placebo. Subjects not using angiotensin converting enzyme inhibitors (ACE-inhibitors) or angiotensin receptor blockers (ARBs) at inclusion were put on ACE-inhibitors for at least two weeks before the baseline measurement and randomisation. Those who were on ARBs continued their ARBs. We excluded individuals with diabetes mellitus (ADA criteria), active vasculitis, nephrotic syndrome (>3g/24h urine protein), renal transplantation, fasting total cholesterol > 7 mmol/L, cholesterol-lowering therapy within three months prior to inclusion or known ischemic cardiac, cerebrovascular or peripheral arterial disease. Ninety-three patients (out of 118 eligible patients) took part in the study and written informed consent was obtained from all participants. Baseline data of these individuals were used for this investigation.

Procedures

All patients were examined in the fasting state in a supine position in a temperature-controlled room. Firstly, data were collected with regard to age, medication and smoking status (having smoked in the past year) and a detailed history was obtained to exclude clinically relevant peripheral, cerebral and coronary vascular disease. Thereafter, height and weight were measured with the individuals wearing light clothing and a thorough physical examination was done. After 30 minutes of rest, blood pressure was measured with an oscillometric device (Colin Press-Mate, model BP-8800, Komaki-City, Japan) and expressed as the mean value of six measurements over a period of 30 minutes. Mean arterial pressure was calculated as (2 diastolic pressure + systolic pressure) / 3. Blood samples were collected after 15 minutes of rest and immediately placed on ice and centrifuged within 15minutes. Plasma samples were stored at –80 °C until analysis.

Carotid ultrasonography

The carotid IMT measurements were performed using Pie Medical Scanner 350 (Pie Medical, Maastricht, The Netherlands) with a linear array transducer of 7.5MHz attached to a data registration and processing unit (Wall Track System II). A single trained operator performed all scans. After 15 minutes of supine rest, subjects were examined with the head turned 30° to the left. B-mode images were obtained of the right common carotid artery at 0.5, 1 and 2 cm proximal to the bulb in the longitudinal plane, after which an M-line was positioned perpendicular to the posterior wall, showing a clear intima-media complex. Subsequently, the system was switched to M-mode and radio-frequency signals were collected in the data acquisition memory of the Wall Track System, which automatically detected and placed markers at the blood-intima interface and media-adventitia interface on the radio-frequency line. Radio-frequency signals of the posterior wall were registered during each heartbeat triggered by the R-top of the simultaneously recorded electrocardiogram. During each measurement, data obtained during four heartbeats were analysed separately; the mean of these four measurements was used. Three measurements each were performed at three locations in the common carotid artery and mean value of these measurements was taken as carotid IMT.

Reproducibility

Reproducibility was assessed in ten healthy subjects (43 ± 13 years) who were examined by the same observer twice, 3 weeks apart. The intra-observer coefficient of variation for the IMT measurement was 10%.

Laboratory analyses

Total cholesterol, HDL cholesterol, and triglycerides were measured by routine laboratory methods. We calculated LDL cholesterol by use of Friedewald formula [18] (two participants had triglyceride levels of >4.5mmol/L and their LDL values were not used in the evaluation). Serum creatinine concentration was assessed by a kinetic Jaffé method. Plasma concentrations of arginine, ADMA and SDMA were determined simultaneously by high-performance liquid chromatography as described previously [19]. In short, sample clean-up was performed by solid-phase extraction on polymeric cation-exchange extraction columns using monomethylarginine as internal standard. After derivatization with ortho-phthaldialdehyde reagent containing 3-mercaptoproprionic acid, analytes were separated

by isocratic reversed-phase high-performance liquid chromatography with fluorescence detection. Analytical recovery was 98-102% and the interassay coefficient of variation was <3%. Plasma ADMA concentration in 53 healthy individuals was 0.42 ± 0.06 μmol/L and SDMA concentration 0.47 ± 0.08 μmol/L [19]. Plasma concentrations of CRP were measured with a highly sensitive in-house ELISA with rabbit anti-CRP (Dako, Copenhagen, Denmark) as a capturing and tagging antibody, with intra- and interassay coefficients of variation of 3.8% and 4.7%, respectively. Soluble vascular cell adhesion molecule-1 (sVCAM-1) was measured by an ELISA method (Diaclone, Besancon, France) with an intra- and interassay coefficient of variation of 4.0 and 8.6%, respectively. Plasma total (free plus protein-bound) homocysteine was measured with an automated fluorescence polarization immunoassay on an Abbot IMx analyzer, with an interassay coefficient of variation <4% [20].
Renal function was estimated by four methods: (1) the serum creatinine level in μmol/L; (2) the Cockcroft-Gault formula in ml/min per 1.73m^2 [21,22]; (3) 24-hour urinary creatinine clearance in ml/min per 1.73m^2; (4) the abbreviated MDRD study equation (estimated GFR in ml/min/1.73 m^2 (186 x $(S_{cr})^{-1.154}$ x $(age)^{-0.203}$ x (0.742 if female) x (1.210 if African)) [23].

Statistical analyses

All analyses were carried out with the SPSS 11.5 software program (SPSS, Chicago, IL, USA). Variables were tested for normality and log transformed if necessary. Pearson's test was used to calculate correlation coefficients. Linear regression analysis was performed to investigate the association between estimates of renal function and plasma ADMA concentration, and the associations between plasma ADMA concentration and carotid IMT, sVCAM-1, and CRP. All associations were first analysed without adjustments and then with adjustments for potential confounders (LDL cholesterol, HDL cholesterol, BMI, smoking, age, mean blood pressure, homocysteine) and expressed in standardized betas. Probability values < 0.05 were considered statistically significant.

Results

Table 1 shows baseline characteristics of the participants. The causes of underlying renal disease in these patients were: hypertension (29 patients), polycystic kidney disease (13 patients), obstructive uropathy (13 patients), glomerulonephritis (11 patients), drug-induced (9 patients), vesico-ureteral reflux (9 patients), IgA nephropathy (2 patients) and miscellaneous (7 patients).

ADMA and renal function

Plasma ADMA concentration exceeded the upper limit of the normal range of 0.54 μmol/L in 34% (32/93) of the patients. In univariate analysis only renal function was significantly related to plasma ADMA (standardized β for Cockcroft-Gault clearance = -0.276, P = 0.007, Figure 1). After adjustments for potential confounders (age, BMI, plasma homocysteine, smoking status, HDL and total cholesterol) creatinine clearance remained the only significant determinant of plasma ADMA concentration (β = -0.342, p = 0.023). Other estimates of renal function gave similar results (data not shown).

ADMA and carotid IMT, CRP and sVCAM-1

Carotid IMT was significantly related to plasma ADMA ($\beta = 0.459$, $P<0.0001$, Figure 2), age ($\beta = 0.407$, $P<0.0001$), mean arterial pressure ($\beta = 0.340$, $P = 0.001$) and HDL cholesterol ($\beta = -0.207$, $P = 0.05$). There was no significant relationship between carotid IMT and other lipid parameters, renal function (any of the described methods), plasma homocysteine, SDMA, sVCAM-1 or CRP (Table 2). After adjustment for BMI, renal function, CRP, LDL and HDL cholesterol, smoking, homocysteine and mean arterial pressure, the only independent determinants of carotid IMT were plasma ADMA ($\beta = 0.445$, $P<0.0001$), age ($\beta = 0.350$, $P<0.0001$) and mean arterial pressure ($\beta = 0.179$, $P = 0.05$).

There was no significant relationship between plasma ADMA and CRP in this population ($\beta= -0.134$, $P=0.204$). Ten subjects had high CRP levels (>15mg/L). When these individuals were excluded, the relationship between CRP and carotid IMT became significant ($\beta = 0.323$, $P = 0.003$). There was no significant relationship between ADMA and CRP in this subgroup. Univariate relationships between ADMA and carotid IMT ($\beta = 0.403$, $P<0.0001$) and age and carotid IMT ($\beta = 0.383$, $P<0.0001$) were also significant in this population. After adjustment for age, BMI, renal function, CRP, LDL and HDL cholesterol, smoking, homocysteine, ADMA and mean blood pressure only ADMA ($\beta = 0.379$, $P<0.0001$), age ($\beta = 0.335$, $P = 0.001$) and CRP ($\beta = 0.212$, $P = 0.04$) were significantly related to carotid IMT. Plasma sVCAM-1 was significantly correlated with plasma ADMA ($\beta = 0.260$, $P= 0.010$) but not with CRP in the total population. In multiple regression analysis with sVCAM-1 as dependent variable and ADMA, age, BMI, smoking, LDL and HDL cholesterol, renal function (any method) and homocysteine as independent variables, only ADMA was significantly related to plasma sVCAM-1 concentration ($\beta = 0.245$, $P = 0.025$). Plasma sVCAM-1 had no significant relationship with carotid IMT.

Plasma SDMA and renal function, homocysteine, and ADMA

Plasma SDMA was strongly related to Cockcroft-Gault creatinine clearance, both in univariate ($\beta = -0.727$, $P <0.0001$) and multiple regression analysis adjusted for age, BMI, total cholesterol and smoking ($\beta = -0.850$ $P<0.0001$). Plasma SDMA was not significantly related to carotid IMT, CRP or sVCAM-1. Plasma SDMA was significantly related to plasma homocysteine ($\beta = 0.511$, $P <0.0001$) but adjustment for renal function (all four methods) weakened this association (β for Cockcroft-Gault creatinine clearance = 0.137, $P = 0.12$).

Discussion

In this population with mild to moderate non-diabetic renal failure, renal function was inversely associated with plasma ADMA. Plasma ADMA, in turn, was strongly and independently associated with carotid IMT and with plasma sVCAM-1, but not with plasma CRP.

Many authors have reported that concentrations of ADMA are increased in patients with renal insufficiency [5,8,24,25,26]. However, many of these studies contained small groups of patients and patients with end stage renal disease. In a study by Fleck *et al*. [27], although the mean ADMA concentration in chronic renal failure patients was 38% higher than in the healthy controls, there was no significant correlation between plasma ADMA and serum creatinine level in patients with chronic renal failure. Kielstein *et al.* [24] reported that ADMA concentrations were significantly higher in patients with renal disease (even in mild renal failure) compared with matched controls without any overlap of ADMA concen-

trations between the two groups. They concluded that increased ADMA levels characterize patients with renal disease as a separate population when compared with normotensive subjects without renal and cardiac disease.

Several observations have linked higher plasma ADMA concentrations to atherosclerosis, cardiovascular events and mortality in subjects who presumably had a normal renal function [28, 29, 30] and in patients with end-stage renal disease [13]. Böger *et al*. [28] showed that ADMA levels correlated strongly with the severity of atherosclerotic disease in individuals with peripheral arterial occlusive disease. In addition, Kielstein *et al*. [12] demonstrated that plasma ADMA concentrations were higher in dialysis patients with clinically manifest atherosclerosis than those without atherosclerosis. The concept of a link between plasma ADMA and atherosclerosis is further strengthened by studies that have shown that plasma ADMA correlates with carotid IMT in apparently healthy middle-aged individuals [29] as well as in dialysis

patients [10]. An important new finding of the present study is that plasma ADMA was directly associated with carotid IMT in patients with mild to moderate renal failure, suggesting that plasma ADMA may be one of the mechanisms that link mild to moderate renal failure with cardiovascular disease [2,3].

We measured plasma markers of inflammation (CRP) and leukocyte-endothelial adhesion (sVCAM-1) to explore whether inflammation and/or leukocyte adhesion to endothelial cells were related to plasma ADMA levels. Plasma CRP was not significantly related to ADMA, although it showed a significant and independent association with carotid IMT in subjects in whom CRP was < 15 mg/l. This suggests that, in individuals with mild to moderate renal failure, the effect of ADMA on IMT is largely unrelated to inflammatory activity insofar as this is reflected by plasma CRP levels.

Plasma sVCAM-1 was significantly associated with ADMA. ADMA is thought to be an important endogenous NO synthase inhibitor, and inhibition of NO synthesis is known to be associated with an increase in endothelial adhesion molecule expression [31,32,33,34]. Therefore, one of the mechanisms that link increased ADMA levels to atherosclerosis in individuals with mild to moderate renal failure may involve upregulation of leukocyte adhesion molecules on endothelial cells. In a recent study, Suda *et al*. [35] demonstrated that coronary microvascular lesions are induced to a comparable extent during long-term administration of ADMA in wild type and eNOS- knockout mice. They also demonstrated an upregulation of ACE and increased oxidative stress through stimulation of angiotensin-1 receptor in both groups. They concluded that long-term vascular effects of ADMA might not solely be mediated by inhibition of endothelial NO synthase. In our population, 76 subjects used ACE-inhibitors or ARBs at inclusion; the others received the ACE-inhibitor fosinopril before inclusion. We did not measure the levels of ACE or oxidative stress parameters. Therefore, we are not able to report on any other probable mechanisms of action of ADMA in our patients. Nevertheless, our results show that the associations of ADMA with carotid IMT and sVCAM-1 are present even during treatment with ACE-inhibitors and ARBs.

Plasma SDMA showed a strong and significant correlation with renal function in our population. In several other studies, the plasma SDMA level was about 3-8 times higher than plasma ADMA level [5,8,24,25,26]. While SDMA is almost exclusively cleared by renal excretion, ADMA is to a significant degree metabolised by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [36,37,38]. This explains why SDMA shows a stronger relationship with renal function than ADMA. The biological significance of SDMA is still

uncertain and a direct inhibitory effect on NO synthase has not been documented.
Metabolic pathways generating homocysteine and ADMA are closely related. ADMA is generated via posttranslational methylation of arginine residues of proteins and these methyl groups probably come from demeythylation process of methionine to homocysteine [38]. Plasma ADMA had no significant relationship with homocysteine in our population and in the multivariate analysis adjustments for homocysteine did not change the significant relationships between ADMA and carotid IMT, ADMA and sVCAM and renal function and ADMA. Plasma ADMA concentration is also raised in young hypercholesterolemic patients with normal renal function [39]. Adjustments for total or HDL cholesterol also did not affect the above mentioned relationships, which therefore were probably independent of hyperhomocysteinemia or dislipidemia.

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Study limitations:

We studied a small and highly selected population of patients with mild to moderate renal failure and the cross sectional nature of the study does not permit any final conclusions with regard to the causality of described associations. The study was also not designed to examine new mechanisms that link increased ADMA levels with atherothrombosis and renal failure. Therefore, we are not able to report on any new mechanisms that explain this probable link. Most of the patients in our study (76 patients) were on long term ACE-inhibitors or ARBs at the start of the study. Recent studies in patients with essential hypertension and diabetes mellitus have revealed that pharmacological treatment with ACE inhibitors may reduce plasma ADMA levels, possibly via enhancement of DDAH activity [40,41]. In addition, it has been demonstrated that treatment with ACE inhibitors is associated with lower plasma CRP levels [42,43]. Therefore, use of ACE inhibitors and ARBs in our population may have negatively influenced the association between plasma CRP and ADMA.

In conclusion, in non-diabetic patients with mild to moderate renal failure without clinical features of atherosclerosis, renal function showed a significant and independent relationship with plasma ADMA levels which, in turn, were associated with carotid IMT and sVCAM-1 levels. The relationship of ADMA with carotid IMT was stronger than the relationship between carotid IMT and age. Plasma ADMA may be one of the mechanisms that link mild to moderate renal failure with cardiovascular disease.

N=93	N(Percentage), mean ± SD, median (range)
Male gender N (%)	53 (57)
Age (yrs)	53 ± 12
Smokers N (%)	30 (36)
BMI (kg/m^2)	26.2 ± 4.8
Blood pressure (mmHg)	
Systolic	136 ± 20
Diastolic	97 ± 11
Mean	79 ± 11
Pulse rate (beats per minute)	57 ± 13
Lipids (mmol/l)	
Total cholesterol	5.2 ± 1.0
HDL	1.3 ± 0.4
LDL	3.2 ± 0.8
Triglycerides	1.7 ± 0.4
Renal function	
Serum creatinine (µmol/L)	205 ± 86
Cockcroft-Gault formula (ml/min/1.73m^2)	38 ± 15
24-hour creatinine-clearance (ml/min/1.73m^2)	41 ± 17
MDRD formula (ml/min/1.73m^2)	35 ± 14
Antihypertensive medication	
ACE inhibitors - no (%)	58 (62)
Angiotensin receptor blockers - no (%)	18 (19)
Diuretics – no (%)	44 (47)
Beta-blockers – no (%)	34 (37)
Alpha-blockers – no (%)	4 (4)
Calcium channel blockers – no (%)	19 (20)
Plasma ADMA (µmol/L)	0.52 ± 0.07
Plasma SDMA (µmol/L)	1.12 ± 0.47
Plasma Arginine (µmol/L)	96.4 ± 17.1
Plasma Homocysteine (µmol/L)	21.3 ± 9.3
Plasma CRP (mg/L)	3.1 (range = 0.1 - 85.2)
Plasma s-VCAM-1 (ng/ml)	959 ± 229
Intima-media thickness (mm)	0.67 ± 0.13

Table 1. Baseline characteristics of the participants

	Standard deviation	Standardized β	p-value
Age	12 years	0.407	<0.0001
ADMA	0.07 µmol/L	0.459	<0.0001
BMI	4.8 kg/m^2	0.178	0.90
LDL-cholesterol	0.82 mmol/L	0.179	0.94
HDL- cholesterol	0.35 mmol/L	-0.207	0.050
Mean blood pressure	16 mmHg	0.340	0.001
CRP	11.1 mg/L	- 0.028	0.792
Creatinine clearance (Cockcroft-Gault formula)	15.7 ml/min/1.73m^2	- 0.131	0.214
Homocysteine	9.3 µmol/L	0.060	0.572
sVCAM-1	229 ng/ml	0.082	0.440
SDMA	0.47 µmol/L	0.050	0.636

Table 2. Univariate associations between carotid IMT and cardiovascular risk factors. The regression coefficients are expressed as standardized β (in mm) per change of 1 standard deviation of the independent variable to facilitate direct comparison.

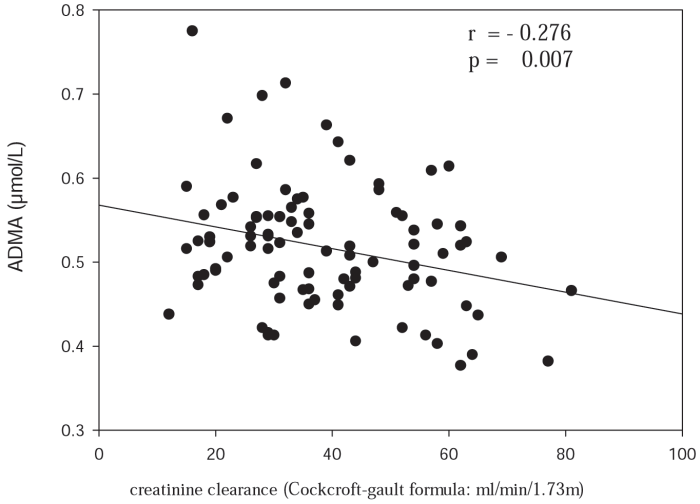

Figure 1. Relationship between plasma ADMA and creatinine clearance (Cockcroft-Gault per 1.73 m^2 body surface area)

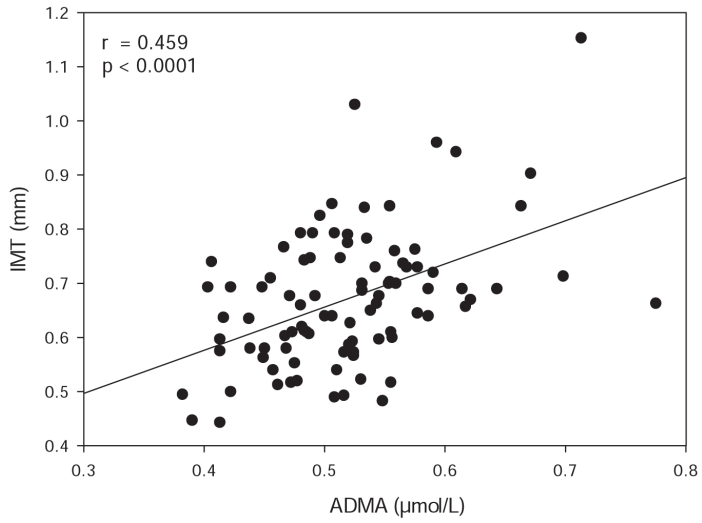

Figure 2. Relationship between plasma asymmetric dimethylarginine (ADMA) and carotid intima-media thickness (IMT)

Reference List

1. Foley RN, Parfrey PS, Sarnak MJ: Epidemiology of cardiovascular disease in chronic renal disease. *J Am Soc Nephrol* 9(Suppl 12):S16-S23, 1998

2. Henry RM, Kostense PJ, Bos G, *et al*: Mild renal insufficiency is associated with increased cardiovascular mortality: The Hoorn Study. *Kidney Int* 62:1402-1407, 2002

3. Muntner P, He J, Hamm L, *et al*: Renal insufficiency and subsequent death resulting from cardiovascular disease in the United States. *J Am Soc Nephrol* 13:745-753, 2002

4. Zoccali C: Cardiovascular risk in uraemic patients-is it fully explained by classical risk factors? *Nephrol Dial Transplant* 15:454-457, 2000

5. Vallance P, Leone A, Calver A, *et al*: Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 339:572-575, 1992

6. Vallance P, Leiper J: Cardiovascular biology of the asymmetric dimethylarginine: dimethylarginine dimethylaminohydrolase pathway. *Arterioscler Thromb Vasc Biol* 24:1023-1030, 2004

7. Kielstein JT, Impraim B, Simmel S, *et al*: Cardiovascular effects of systemic nitric oxide synthase inhibition with asymmetrical dimethylarginine in humans. *Circulation* 109:172-177, 2004

8. MacAllister RJ, Rambausek MH, Vallance P, *et al*: Concentration of dimethyl-L-arginine in the plasma of patients with end-stage renal failure. *Nephrol Dial Transplant* 11:2449-2452, 1996

9. Zoccali C, Benedetto FA, Maas R, *et al*: Asymmetric dimethylarginine, C-reactive protein, and carotid intima-media thickness in end-stage renal disease. *J Am Soc Nephrol* 13:490-496, 2002

10. Hodis HN, Mack WJ, LaBree L, *et al*: The role of carotid arterial intima-media thickness in predicting clinical coronary events. *Ann Intern Med* 128:262-269, 1998

11. Benedetto FA, Mallamaci F, Tripepi G, Zoccali C: Prognostic value of ultrasonographic measurement of carotid intima media thickness in dialysis patients. *J Am Soc Nephrol* 12:2458-2464, 2001

12. Kielstein JT, Böger RH, Bode-Böger SM, *et al*: Asymmetric dimethylarginine plasma concentrations differ in patients with end-stage renal disease: relationship to treatment method and atherosclerotic disease. *J Am Soc Nephrol* 10:594-600, 1999

13. Zoccali C, Bode-Böger S, Mallamaci F, *et al*: Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet* 358:2113-2117, 2001

14. Stam F, van Guldener C, Schalkwijk CG, *et al*: Impaired renal function is associated with markers of endothelial dysfunction and increased inflammatory activity. *Nephrol Dial Transplant* 18:892-898, 2003

15. Arici M, Walls J: End-stage renal disease, atherosclerosis, and cardiovascular mortality: is C-reactive protein the missing link? *Kidney Int* 59:407-414, 2001

16. Zoccali C, Benedetto FA, Mallamaci F, *et al*: Inflammation is associated with carotid atherosclerosis in dialysis patients. Cardiovascular Risk Extended Evaluation in Dialysis Patients. *J Hypertens* 18:1207-1213, 2000

17. Haubitz M, Brunkhorst R: C-reactive protein and chronic Chlamydia pneumoniae infection long-term predictors for cardiovascular disease and survival in patients on peritoneal dialysis. *Nephrol Dial Transplant* 16:809-815, 2001

18. Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18:499-502, 1972

19. Teerlink T, Nijveldt RJ, de Jong S, van Leeuwen PA: Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. *Anal Biochem* 303:131-137, 2002

20. Shipchandler MT, Moore EG: Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyser. *Clin Chem* 41:991-994, 1995

21. Cockcroft DW, Gault MH: Prediction of creatinine clearance from serum creatinine. *Nephron* 16:31-41, 1976

22. Du Bois D, Du Bois EF: A formula to estimate the approximate surface area if height and weight be known. 1916. Nutrition 5: 303-311, 1989

23. NATIONAL KIDNEY FOUNDATION: K/DOQI clinical practice guidelines for chronic kidney disease: Evaluation, classification and stratification: Guideline 4: Estimation of GFR. *Am J Kid Dis* 39(Suppl 1):S83-S86, 2002

24. Kielstein JT, Böger RH, Bode-Böger SM, *et al*: Marked increase of asymmetric dimethylarginine in patients with incipient primary chronic renal disease. *J Am Soc Nephrol* 13:170-176, 2002

25. Pettersson A, Uggla L, Backman V: Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 692:257-262, 1997

26. Wahbi N, Dalton RN, Turner C, *et al*: Dimethylarginines in chronic renal failure. *J Clin Pathol* 54:470-473, 2001

27. Fleck C, Schweitzer F, Karge E, *et al*: Serum concentrations of asymmetric (ADMA) and symmetric (SDMA) dimethylarginine in patients with chronic kidney diseases. *Clin Chim Acta* 336:1-12, 2003

28. Böger RH, Bode-Böger SM, Thiele W, *et al*: Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. *Circulation* 95:2068-2074, 1997

29. Miyazaki H, Matsuoka H, Cooke JP, *et al*: Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. *Circulation* 99:1141-1146, 1999

30. Valkonen VP, Paiva H, Salonen JT, *et al*: Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine. *Lancet* 358:2127-2128, 2001

31. Cooke JP: Does ADMA cause endothelial dysfunction? *Arterioscler Thromb Vasc Biol* 20:2032-2037, 2000

32. Theilmeier G, Chan JR, Zalpour C, *et al*: Adhesiveness of mononuclear cells in hypercholesterolemic humans is normalized by dietary arginine. *Arterioscler Thromb Vasc Biol* 17:3557-3564, 1997

33. Goonasekera CD, Shah V, Rees DD, Dillon MJ: Vascular endothelial cell activation associated with increased plasma asymmetric dimethyl arginine in children and young adults with hypertension: a basis for atheroma. *Blood Press* 9:16-21, 2000

34. Maxwell AJ: Mechanisms of dysfunction of the nitric oxide pathway in vascular diseases. *Nitric oxide* 6:101-124, 2002

35. Suda O, Tsutsui M, Morishita T, *et al*: Asymmetric dimethylarginine produces vascular lesions in endothelial nitric oxide synthase-deficient mice: involvement of reninangiotensin system and oxidative stress. *Arterioscler Thromb Vasc Biol* 24:1682-1688, 2004

36. Leiper JM, Santa Maria J, Chubb A, *et al*: Identification of two human dimethylarginine demethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases. *Biochem J* 343:209-214, 1999

37. Ogawa T, Kimoto M, Sasaoka K: Purification and properties of a new enzyme, NG, NG-dimethylarginine dimethylaminohydrolase, from rat kidney. *J Biol Chem* 264:10205-10209, 1989

38. Böger RH, Bode-Böger SM, Sydow K, *et al*: Plasma concentration of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, is elevated in monkeys with hyperhomocyst(e)inemia or hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 20:1557-1564, 2000

39. Böger RH, Bode- Böger SM, Szuba A, *et al*: Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction. Its role in hypercholesterolemia. *Circulation* 98:1842-1847, 1998

40. Ito A, Egashira K, Narishige T, *et al*: Angiotensin-converting enzyme activity is involved in the mechanism of increased endogenous nitric oxide synthase inhibitor in patients with type 2 diabetes mellitus. *Circ J* 66:811-815, 2002

41. Chen JW, Hsu NW, Wu TC, *et al*: Long-term angiotensin-converting enzyme inhibition reduces plasma asymmetric dimethylarginine and improves endothelial nitric oxide bioavailability and coronary microvascular function in patients with syndrome X. *Am J Cardiol* 90:974-982, 2002

42. Di Napoli M, Papa F: Angiotensin-converting enzyme inhibitor use is associated with reduced plasma concentration of C-reactive protein in patients with first-ever ischemic stroke. *Stroke* 34:2922-2929, 2003

43. Takeda T, Hoshida S, Nishino M, *et al*: Relationship between effects of statins, aspirin and angiotensin II modulators on high-sensitive C-reactive protein levels. *Atherosclerosis* 169:155-158, 2003

Chapter 5

Association between global leukocyte DNA methylation, renal function, carotid intima-media thickness and plasma homocysteine in patients with stage 2-4 chronic kidney disease

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Abstract

Background
Patients with chronic kidney disease (CKD) have an increased risk of cardiovascular disease (CVD). Preliminary evidence suggest a role for global DNA hypomethylation in the pathogenesis of atherosclerotic complications in CKD. The aims of this study in patients with stage 2-4 CKD were: 1) To assess the association between renal function and DNA methylation, 2) To assess the association between DNA methylation and two markers of atherosclerosis (common carotid intima-media thickness (CCA-IMT) and brachial artery endothelium-dependent, flow-mediated dilatation (BA-FMD), and 3) To examine the effect of a multi-step treatment strategy on DNA-methylation .

Methods
In the Anti-oxidant Therapy In Chronic renal insufficiency study (ATIC-study), 93 patients with stage 2-4 CKD were included. In a randomised, double blind, placebo-controlled design, the treatment group received pravastatin to which vitamin E was added after 6 months and homocysteine-lowering B-vitamin therapy after another 6 months. DNA methylation was assessed using tandem-mass-spectrometry. CCA-IMT and BA-FMD were assessed using B-mode ultrasonagraphy.

Results
At baseline, global DNA methylation was not associated with estimated glomerular filtration rate (P=0.32) or with CCA-IMT (P=0.62) or BA-FMD (P=0.51). No effect of the treatment strategy including B-vitamins on global DNA methylation was found either in the total study group or within separate strata of homocysteine concentration and renal function.

Conclusion
In patients with stage 2-4 CKD, global DNA methylation is not associated with renal function or with CCA-IMT or BA-FMD. A treatment strategy which includes B-vitamins did not alter global DNA methylation in these patients. These data do not support a role for DNA hypomethylation in CKD-associated vascular disease in patients with stage 2-4 CKD.

Introduction
Chronic kidney disease (CKD) is associated with an increased incidence of cardiovascular disease (CVD). When the glomerular filtration rate (GFR) decreases below the 70ml/min, the probability of CVD increases markedly[1]. This increase is not fully explained by traditional cardiovascular risk factors.
Recently, impaired one-carbon metabolism (figure 1)[2-4] has been recognized as one of the possible mechanisms responsible for increased atherogenicity in CKD patients. In patients with end stage renal disease (ESRD), an elevated homocysteine concentration with impaired transsulfuration and remethylation of homocysteine, and elevated S-adenosylhomocysteine (SAH) levels, has been demonstrated [5;6] (figure 1). Increased SAH inhibits methyltransferases, leading to impairment of methylation reactions[7]. Global DNA hypomethylation has been demonstrated in dialysis patients[8] and is implicated as an important candidate contributor to CVD in patients with ESRD[9]. However, even prior to ESRD, both CVD incidence and plasma homocysteine concentrations rise sharply in proportion to

the loss of renal function[6]. Whether DNA hypomethylation is also a feature of these earlier stages of renal insufficiency is currently unknown.
Global DNA hypomethylation has been associated with various diseases including atherosclerotic vascular disease[10]. Low global DNA methylation has been shown in atherosclerotic arteries of animals[11] and humans[12]. Moreover, in a small case-control study, patients with vascular disease had lower leukocyte global DNA methylation compared to healthy controls[13]. However, data in support of an association between DNA hypomethylation and vascular disease in patients with mild to moderate kidney disease are not available and potential mechanisms that might explain such an association have not been studied.
In view of these considerations, we designed this study to assess in a population of stage 2-4 CKD patients the relationship between i) renal function and DNA methylation, ii) DNA methylation and two established surrogate markers of arterial vascular disease: common carotid artery intima media thickness (CCA-IMT)[14] and brachial artery flow-mediated endothelial dependant vasodilatation (BA-FMD)[15] and iii) to assess the effect on DNA methylation of a stepwise treatment strategy which includes B-vitamins.

Methods
This study is a part of the Anti-oxidant Therapy In Chronic renal insufficiency study (ATIC-study); a randomised, double blind, placebo-controlled clinical trial, which was performed to investigate the effect of a treatment strategy designed primarily to achieve a stepwise oxidative stress reduction on vascular structure and function in patients with CKD[16]. Between May 2001 and December 2002, CKD patients (n=700) with a creatinine clearance of 15-70 ml / min per 1.73m^2 (according to the Cockcroft-Gault equation[17]) from 7 outpatient clinics in Amsterdam, The Netherlands, were screened for eligibility for participation.
Patients with diabetes mellitus, active vasculitis, nephrotic syndrome, renal transplantation, hypercholesterolaemia (>7 mmol/L), cholesterol-lowering therapy within the past 3 months or a history of cardiovascular disease were excluded from the study. Out of 118 eligible patients, 93 gave informed consent. Baseline data of these individuals were used to assess the association between global leukocyte DNA methylation on the one hand and renal function, CCA-IMT and BA-FMD on the other hand. Longitudinal data were used to assess the treatment effects on DNA methylation.

Baseline measurements
Data were collected with regard to age, medication and smoking status (having smoked in the past year). A detailed history was obtained to exclude clinically relevant peripheral, cerebral and coronary vascular disease at baseline. Height and weight were measured with the individuals wearing light clothing.
Blood samples were taken after an overnight fast. Global DNA methylation was measured by liquid chromatography- tandem mass spectrometry as described in detail by Kok *et al*[18]. In short, DNA was isolated from leukocytes and 1 μg of genomic DNA was hydrolyzed using formic acid. Cytosine (Cyt) and 5-methylcytosine (mCyt) were separated using gradient-elution reversed phase chromatography with a mobile phase containing 5 mmol/L nonafluoropentanoic acid as ion-paring reagent. Cyt and mCyt were detected by liquid chromatography electro spray ionization tandem mass spectrometry operating in the multiple reaction monitoring mode and quantified using stable isotope dilution. The level of DNA methylation is expressed as the methylcytosine/total-cytosine ratio (mCyt:tCyt).

The intra- and inter-assay coefficients of variation (CV) for the 5-methylcytosine/total cytosine ratio (mCyt:tCyt) was 1.7% (n=9) and 3.5% respectively (n=8) for calf thymus DNA (mean mCyt/tCyt ratio 6.5%), and 4.5% (n=6) and 6.5% (n=14), respectively for *Escherichia coli* pBR322 DNA (mean mCyt/tCyt ratio 0.48%). The mean intra-and inter-assay CV's for humans (n=10) was 1.4 and 4.1% respectively.

Renal function was estimated by The Modification of Diet in Renal Disease (MDRD) study equation (estimated GFR in ml/min/1.73 m^2) : 170 x [plasma creatinine μmol/L x 0.0113] $^{-0.999}$ x [age] $^{-0.176}$ x [plasma urea mmol/L x 2.8] $^{-0.170}$ x [Albumin g/L x 0.1] $^{+0.318}$ x [0.762 if female] x [1.18 if black] as described in detail elsewhere[19].

Plasma total (free plus protein-bound) homocysteine was measured with an automated fluorescence polarization immunoassay on an Abbott IMx analyzer (Abbott Laboratories, Abbott Park, IL, USA), with an interassay coefficient of variation <4%[20].

Plasma concentrations of CRP were measured with a highly sensitive in-house enzyme-linked immunosorbent assay (ELISA) with rabbit anti-CRP (Dako, Copenhagen, Denmark) as a capturing and tagging antibody, with intra- and interassay coefficients of variation of 3.8% and 4.7%, respectively.

The 677C>T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene has been suggested to affect global DNA methylation under specific conditions, and was therefore assessed by polymerase chain reaction[21].

Vascular measurements were taken after acclimatisation in a temperature controlled (25 °C) room. The common carotid artery intima-media thickness (CCA-IMT) measurements were performed using a Pie Medical Scanner 350 (Pie Medical, Maastricht, The Netherlands) with a linear array transducer of 7.5MHz attached to a data registration and processing unit (Wall Track System II), as described elsewhere[16]. The measurement protocol for the brachial artery endothelium-dependent, flow-mediated dilatation (BA-FMD) has also been described in detail elsewhere[16]. Briefly, baseline diameter (mean of 3 measurements) and peak flow velocity (mean of 2 measurements) were determined. After release of the pressure cuff, maximum peak flow velocity was measured within 15 seconds and diameter was measured at 45, 90, 120, 150 and 300 seconds. All ultrasound measurements were performed by a single trained operator and assessed at 0, 6, 12 and 18 months.

Intervention

The study design was described in detail elsewhere[16]. After randomization, participants in the active treatment group were treated with pravastatin 40 mg/day for six months. Subsequently vitamin E 300 mg/day (450 IU α-tocopherolacetate) was added for another six months and lastly homocysteine-lowering therapy (folic acid 5 mg/day, pyridoxine 100 mg/day and vitamin B12 1 mg/day all in one tablet) was added for another 12 months. The total duration of the study was 24 months. Patients in the placebo group received matching placebo tablets at the onset and 6 and 12 months later. In order to establish whether this treatment strategy had any effect on DNA-methylation, the mCyt:tCyt ratio in leukocyte DNA was reassessed at 6-monthly intervals during this study period.

Statistical analysis

Statistical analysis was carried out with Stata Statistical Software for windows, release 7. Linear regression analysis was performed at baseline to investigate primarily the association between global DNA methylation (mCyt:tCyt ratio) on the one hand and renal function,

CCA-IMT and BA-FMD on the other hand. Additional analyses were performed after stratification into tertiles of baseline values of renal function, and homocysteine concentration. Stratified analysis was also performed within MTHFR 677 C>T categories. Subjects with a CRP concentration >10 mg/L during the study were excluded in the analysis because inflammation may influence leukocyte DNA methylation, as was recently suggested[22].
Patients continuing trial participation after the baseline measurements were analyzed according to intention-to-treat. The treatment effect was determined by performing generalized estimating equations (GEE) with mCyt:tCyt ratio as dependent variable. The primary independent variable in the GEE model was treatment strategy (1= intervention group, 0=placebo group) adjusted for time and baseline mCyt:tCyt ratio. To assess the effect at the different time points, time was treated as a categorical variable and represented by dummy variables. The GEE analysis assesses the relationship between the treatment modalities and mCyt:tCyt ratio by correction for the within subject's dependency as a result of the repeated observations[23]. A p-value less than 0.05 was considered as significant.

Results

Out of 93 included patients, 15 were excluded because of an elevated CRP level, leaving 78 subjects for analysis. Baseline characteristics of these patients are reported in Table 1. At baseline, the mCyt:tCyt ratio ranged from 3.70 to 5.43% (median: 4.41%). In comparison, the normal range, identified in healthy subjects aged 18-62 years with the same analytical method was 2.57 - 4.81% (median: 4.10%)[18].
We observed no association between DNA methylation and age (ß= -0.003; P=0.36), sex (ß=-0.03; P=0.70), BMI (ß=-0.007; P=0.34), smoking status (ß=-0.015; P=0.83) and CRP concentration (ß=-0.004; P=0.73). Stratification according to the MTHFR 677C>T genotype did not change these results (data not shown).

Global leukocyte DNA methylation and renal function

There was no association between DNA methylation and renal function in univariate regression analysis (ß= -0.002; P=0.32). No difference in DNA methylation was found between the highest or second tertile of renal function compared to the lowest tertile (figure 2). Additional analysis within strata of MTHFR 677C>T genotype and tertiles of homocysteine concentrations did not influence these results.

Global leukocyte DNA methylation and homocysteine concentrations

Global DNA methylation was not associated with homocysteine concentrations in univariate analyses (ß= 0.003; P=0.46). No difference was found in DNA methylation between the highest or second tertile of homocysteine concentration compared to the lowest tertile (figure 3). We found no significant difference in DNA methylation in subjects with the MTHFR 677 TT and CT genotype compared to MTHFR 677 CC genotype (ß=0.19; P=0.07 and ß=0.06; P=0.44 respectively). The lack of association between DNA methylation and homocysteine concentration was also confirmed in the separate strata of the MTHFR 677 C>T genotype.

Global leukocyte DNA methylation and carotid intima media thickness and flow-mediated dilatation of the brachial artery

In univariate analysis, we found no association between DNA methylation and CCA-IMT (ß= -0.13; P=0.62) and BA-FMD (ß= 0.007; P=0.51). Adjustment for sex, age, BMI, smoking status, renal

function, CRP and homocysteine concentration weakened this association further (ß= -0.004; P=0.99 and ß= 0.003; P=0.78, respectively).
No difference in DNA methylation was found within strata of CCA-IMT and BA-FMD (figures 4 and 5). Analysis within strata of renal function, homocysteine and the MTHFR 677 C>T genotype did not change these results.

Effect of B-vitamin-containing treatment on DNA methylation
Out of 93 included patients, six withdrew after the baseline measurements and 87 underwent at least one of the follow-up measurements. 15 subjects were excluded because of an elevated CRP level during the study (leaving n=38 and n=39 in placebo and treatment group, respectively).
Overall, we found no significant effect of the treatment strategies, including B-vitamins, on DNA methylation (ß= -0.001; P=0.96; figure 6). No effect of the intervention was found within strata of DNA methylation, renal function and homocysteine at baseline.

Discussion
The main findings of this study are that in stage 2-4 CKD patients global DNA methylation is i) not associated with renal function or homocysteine concentration, (ii) not associated with established markers of atherosclerosis (CCA-IMT) and endothelial function (BA-FMD) and iii) not altered by a treatment strategy which included B-vitamins.
These results are significant since they contradict several findings regarding global DNA methylation in previous small-scale studies.

DNA methylation, renal function, and homocysteine
Some years ago, Ingrosso *et al*[8] demonstrated leukocyte DNA hypomethylation in dialysis patients. A later study failed to show a relationship between renal function and DNA methylation both in stage 3-5 CKD patients and in ESRD patients receiving dialysis[22].
Combined with our results, it thus appears that leukocyte DNA hypomethylation is not a feature of mild to moderate renal failure. Leukocyte DNA hypomethylation may have occurred in the original Ingrosso study in haemodialysis patients due to leukocyte activation on dialysis membranes, and renal failure per se may not play a significant role. Additional studies are needed to elucidate this further.
High plasma homocysteine level is thought to be associated with DNA hypomethylation.
An association between homocysteine and DNA methylation was also demonstrated in haemodialysis patients by Ingrosso *et al*[8] but we could not reproduce this finding in stage 2-4 CKD. Our results are in line with a recent report by Stenvinkel *et al* in CKD patients[22]. Also in another study, no association was demonstrated between homocysteine concentration and DNA methylation in healthy subjects aged 18-62 years[18]. Our findings thus do not support the concept of DNA hypomethylation being responsible for hyperhomocysteinaemia-associated vascular damage, at least not in stage 2-4 CKD.

Global DNA leukocyte methylation and arterial wall properties
Preliminary evidence suggests that global DNA hypomethylation is involved in atherogenesis[2]. However, most of these studies were carried out in animals using DNA methylation from plaques whereas evidence in humans using leukocyte DNA methylation is marginal. Only a single small case control study reported lower leukocyte DNA methylation in CVD

patients compared to age and sex matched healthy controls[13].
We did not observe an association between leukocyte DNA methylation and two strong surrogate markers of CVD. Although this does not exclude a role for DNA methylation in atherothrombotic disease, it does suggest that intima-media thickening and endothelial vasomotor dysfunction are not features of DNA-hypomethylation-associated arterial vascular disease.

Effect of B-vitamin treatment on global DNA leukocyte methylation

We anticipated that the B-vitamins, in particular folic acid, could increase the level of DNA methylation because we previously demonstrated that these vitamins effectively lowered homocysteine concentration in our population (from 20.16 ± 6.80 to 10.45 ± 4.02 μmol/L, versus no significant difference in the placebo group)[16]. Both an increased remethylation rate as well as a decrease in homocysteine (which converts intracellularly to the methylation inhibitor SAH; figure 1) are conceivable mechanisms for such effect of B-vitamins. Ingrosso *et al*[8] indeed showed reversal of DNA hypomethylation after folate treatment. However, a recent study showed no association between B-vitamin status and DNA methylation[18]. Noteworthy is that Ingrosso *et al*[8] used short-term very high-dose folate treatment (15 mg 5-Methylenetetrahydrofolate) in patients with confirmed DNA hypomethylation.

Study limitations

An important general limitation of global leukocyte DNA methylation is in the proper interpretation of what is really measured. Firstly, the extent to which variability in global DNA methylation reflects variability in epigenetic regulation of gene expression, or variability in methylation of non-coding, repetitive DNA regions, is unknown. Also, the degree to which leukocyte DNA methylation reflects the level of DNA methylation in other tissues, for example vascular tissue, is undetermined. Finally, we do not know to what extent DNA methylation reflects methylation of other, potentially more relevant molecules, such as proteins, enzymes, lipoproteins, etc.
One can argue that the cut-off point to define inflammation is high (CRP >10 mg/L). Stenvinkel *et al*. demonstrated a linear association between CRP levels and DNA-methylation only in patients with CRP > 10 mg/L, while in patients with lower CRP levels there was no linear association between CRP levels and DNA methylation[22]. Marked inflammation, as evidenced by CRP >10 mg/L, arguably affects leukocyte metabolism and turnover rate, which may in turn affect leukocyte DNA methylation. As we intended to focus on the effect of renal function on methylation, we thus excluded patients with CRP >10 in our analysis. To prevent subtle confounding by low-grade inflammation in this study, adjustment for CRP concentration was performed.
Limitations specific to this study include the relatively small sample size in particular in subgroup analyses of strata of continuous variables and of the MTHFR 677 C>T genotype. As the TT genotype renders individuals more susceptible to DNA hypomethylation, for example in response to low folate status[24], an association between renal function and DNA methylation in MTHFR 677TT subjects can thus not be excluded. With respect to the intervention part, our study had a power of 80% (at an alpha of 0.05) to detect a 0.13% absolute difference in DNA methylation. We previously found the smallest real difference in DNA methylation using the precise tandem-mass-spectrometry technique to be 0.11%[18]: a difference we thus would have been able to detect in the present study. Finally, stage 5 CKD

patients were not represented in our study, thus precluding conclusions on DNA methylation in more severe renal failure.

Conclusion

In conclusion, in non-diabetic patients with mild-to-moderate chronic kidney disease without clinical features of atherosclerosis, global DNA methylation was not associated with renal function, with homocysteine concentration and with arterial wall properties. A treatment strategy which includes B-vitamins did not alter global DNA methylation in these patients. Our study indicates that DNA hypomethylation is not a feature of mild-to-moderate kidney disease, and is not a likely contributor to accelerated atherosclerosis in renal patients.

	N(%), mean ± SD, *median (range)
Male gender (N (%))	43 (55)
Age (years)	53 ± 10
BMI (kg/m^2)	26 ± 5
Smoking (N(%))	28 (36%)
Use of ACE inhibitors (N (%))	66 (85%)
Plasma homocysteine (µmol/l)	*19.8 (8.7-55.4)
C-reactive protein (mg/l)	*2.0 (0.1-9.9)
Renal function: MDRD formula (ml/min/1.73m^2)	33 ± 14
DNA methylation: methylated cytosine : total cytosine ratio (%)	*4.41 (3.70-5.16)
MTHFR 677 CC/CT/TT genotype (N(%))	28(37%)/37(49%)/11(14%)
CCA-IMT (mm)	0.64 ± 0.13
BA-FMD (%)	4.91 (-1.77-15.06)

Table 1: Baseline characteristics of the participants (N=78)

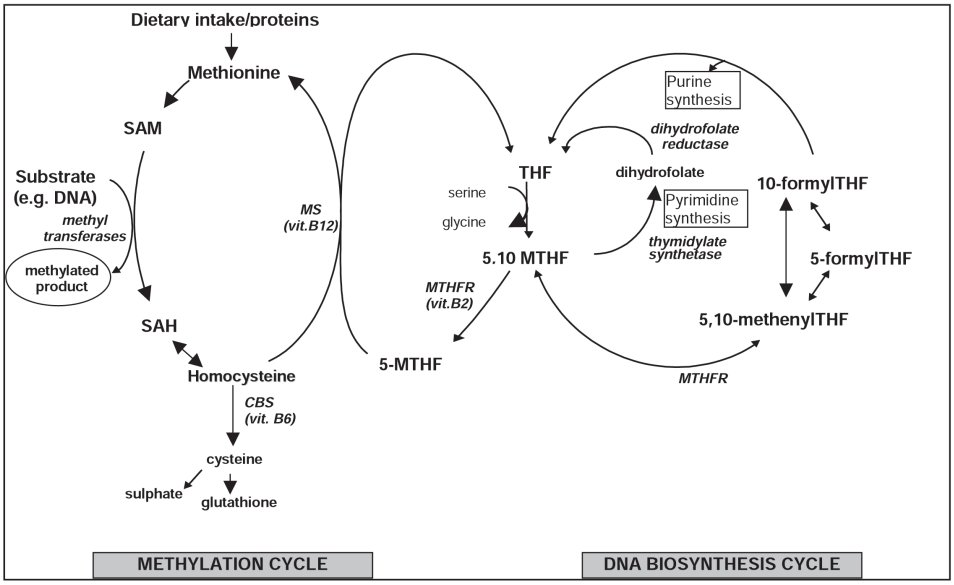

Figure 1: One-carbon metabolism

Methionine derived from the diet, protein breakdown or from remethylation of homocysteine forms S-adenosylmethionine (SAM or Ado Met). SAM can donate a methyl group by methyltransferases to the carbon 5' position of cytosine within the cytosine-guanine dinucleotide for DNA hypomethylation. The demethylated product of SAM, S-adenosylhomocysteine (SAH or Ado Hcy), is hydrolyzed further to homocysteine and adenosine (transmethylation). To prevent accumulation of homocysteine, it has to be hydrolyzed into cysteine by cysthationine-â-synthase (CBS; transsulfuration) and leave the human body, or can be remethylated by a methyl-group of 5-methyltetrahydrofolate (5, MTHF) back into methionine.

(SAM; S-adenosylmethionine, SAH; S-adenosylhomocysteine, CBS; cystathionine ß-synthase, MTHF; methylenetetrahydrofolate, THF; tetrahydrofolate, MTHFR: 5,10 methylenetetrahydrofolate reductase, MS; methionine synthase).

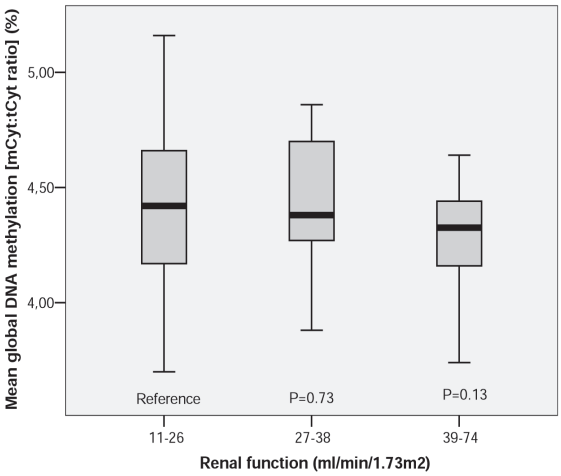

Figure 2: Global DNA methylation in tertiles of estimated glomerular filtration rate (N=78).

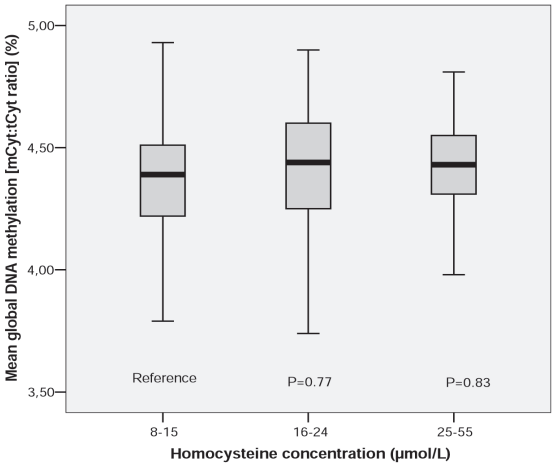

Figure 3: Global DNA methylation in tertiles of homocysteine concentration (N=78).

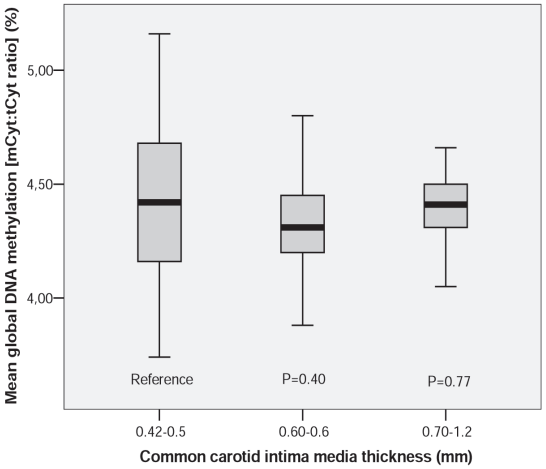

Figure 4: Global DNA methylation by tertiles of CCA-IMT (N=78).

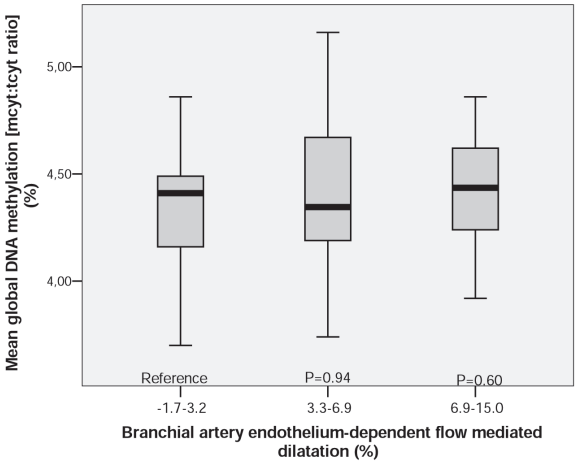

Figure 5: Global DNA methylation by tertiles of BA-FMD (N=78).

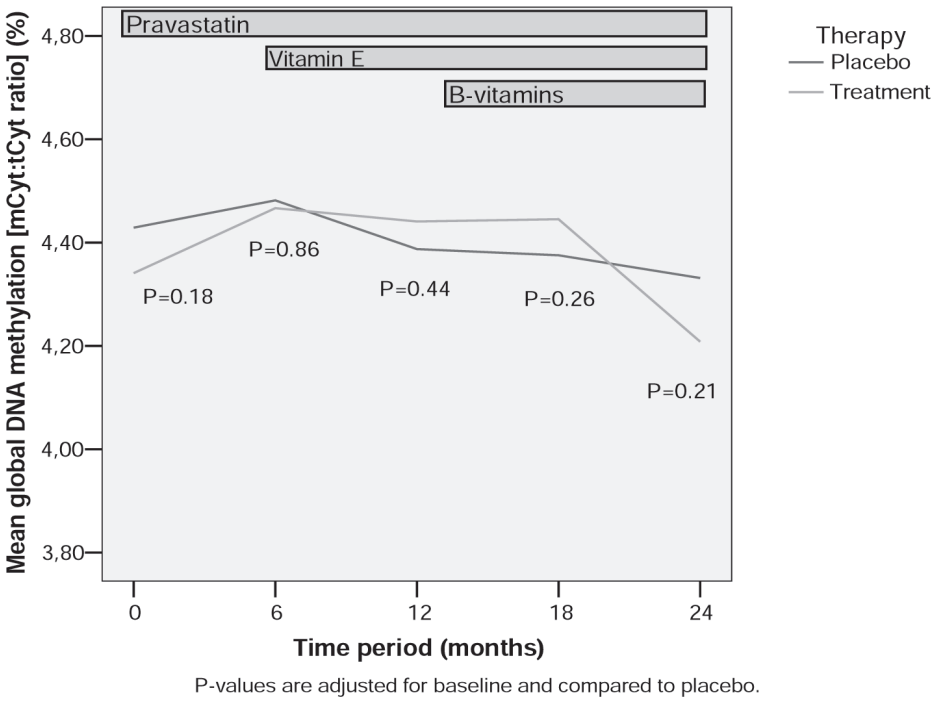

Figure 6: Mean mCyt:tCyt ratio (%) following treatment with placebo; or with pravastatin, vitamin E and B-vitamins (N=78).

Reference List

1. Manjunath G, Tighiouart H, Coresh J *et al*. Level of kidney function as a risk factor for cardiovascular outcomes in the elderly. *Kidney Int* 2003; 63: 1121-1129

2. Castro R, Rivera I, Blom HJ, Jakobs C, Tavares dA, I. Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview. *J Inherit Metab Dis* 2006; 29: 3-20

3. Perna AF, Ingrosso D, Lombardi C *et al*. Possible mechanisms of homocysteine toxicity. *Kidney Int Suppl* 2003; S137-S140

4. van Guldener C, Stam F, Stehouwer CD. Hyperhomocysteinaemia in chronic kidney disease: focus on transmethylation. *Clin Chem Lab Med* 2005; 43: 1026-1031

5. Loehrer FM, Angst CP, Brunner FP, Haefeli WE, Fowler B. Evidence for disturbed S-adenosylmethionine : S-adenosylhomocysteine ratio in patients with end-stage renal failure: a cause for disturbed methylation reactions? *Nephrol Dial Transplant* 1998; 13: 656-661

6. Mallamaci F, Zoccali C, Tripepi G *et al*. Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. *Kidney Int* 2002; 61: 609-614

7. Barber JR, Clarke S. Inhibition of protein carboxyl methylation by S-adenosyl-L-homocysteine in intact erythrocytes. Physiological consequences. *J Biol Chem* 1984; 259: 7115-7122

8. Ingrosso D, Cimmino A, Perna AF *et al*. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. *Lancet* 2003; 361: 1693-1699

9. Perna AF, Capasso R, Lombardi C, Acanfora F, Satta E, Ingrosso D. Hyperhomocysteinemia and macromolecule modifications in uremic patients. *Clin Chem Lab Med* 2005; 43: 1032-1038

10. Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. *Biochim Biophys Acta* 2007; 1775: 138-162

11. Laukkanen MO, Mannermaa S, Hiltunen MO *et al*. Local hypomethylation in atherosclerosis found in rabbit ec-sod gene. *Arterioscler Thromb Vasc Biol* 1999; 19: 2171-2178

12. Castro R, Rivera I, Martins C *et al*. Intracellular S-adenosylhomocysteine increased levels are associated with DNA hypomethylation in HUVEC. *J Mol Med* 2005; 83: 831-836

13. Castro R, Rivera I, Struys EA *et al*. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. *Clin Chem* 2003; 49: 1292-1296

14. Aminbakhsh A, Mancini GB. Carotid intima-media thickness measurements: what defines an abnormality? A systematic review. *Clin Invest Med* 1999; 22: 149-157

15. Gokce N, Keaney JF, Jr., Hunter LM *et al*. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol* 2003; 41: 1769-1775

16. Nanayakkara PW, van Guldener C, ter Wee PM *et al*. Effect of a treatment strategy consisting of pravastatin, vitamin E, and homocysteine lowering on carotid intima-media thickness, endothelial function, and renal function in patients with mild to moderate chronic kidney disease: results from the Anti-Oxidant Therapy in Chronic Renal Insufficiency (ATIC) Study. *Arch Intern Med* 2007; 167: 1262-1270

17. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; 16: 31-41

18. Kok RM, Smith DE, Barto R *et al*. Global DNA methylation measured by liquid chromatography-tandem mass spectrometry: analytical technique, reference values and determinants in healthy subjects. *Clin Chem Lab Med* 2007; 45: 903-911

19. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999; 130: 461-470

20. Shipchandler MT, Moore EG. Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyzer. *Clin Chem* 1995; 41: 991-994

21. Frosst P, Blom HJ, Milos R *et al*. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; 10: 111-113

22. Stenvinkel P, Karimi M, Johansson S *et al*. Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease? *J Intern Med* 2007; 261: 488-499

23. Twisk JW. Longitudinal data analysis. A comparison between generalized estimating equations and random coefficient analysis. *Eur J Epidemiol* 2004; 19: 769-776

24. Friso S, Girelli D, Trabetti E *et al*. The MTHFR 1298A>C polymorphism and genomic DNA methylation in human lymphocytes. *Cancer Epidemiol Biomarkers Prev* 2005; 14: 938-943

Chapter 6

Effect of a Treatment Strategy Consisting of Pravastatin, Vitamin E, and Homocysteine Lowering on Carotid Intima-Media Thickness, Endothelial Function, and Renal Function in Patients With Mild to Moderate Chronic Kidney Disease

Results From the Anti-Oxidant Therapy in Chronic Renal Insufficiency (ATIC) Study

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Abstract

Background

Patients with chronic kidney disease have an increased risk of cardiovascular disease. Oxidative stress has been proposed to play a role in the development of cardiovascular disease among these patients.

Methods

We conducted a randomized, double-blind trial in 93 patients (Cockcroft-Gault equation: creatinine clearance, 38 ± 15 [mean ± SD] mL/min per 1.73 m^2 [0.63 ± 0.25 mL/s per m^2]) to investigate the effect of a treatment strategy designed primarily to achieve stepwise oxidative stress reduction on common carotid intima-media thickness (CC-IMT), brachial artery flow-mediated dilatation (BA-FMD), albuminuria, and renal function. The treatment group received a regimen of pravastatin to which vitamin E supplementation was added after 6 months and homocysteine-lowering therapy after another 6 months. Blood pressure in both groups was managed according to a standard protocol. The placebo group received matching placebos. Measurement of CC-IMT and BA-FMD was performed at randomization after 6, 12, and 18 months. Patients were followed up for 2 years. Generalized estimating equations were used for analysis.

Results

Compared with placebo, active treatment was associated with a decrease in CC-IMT (after 18 months: from 0.68 to 0.63 mm in the treatment group and from 0.65 to 0.71 mm in the placebo group; *P*<.001), an increase in BA-FMD (after 18 months: from 4.66% to 7.56% in the treatment group and from 6.21% to 4.73% in the placebo group; *P*<.001), and an attenuated increase in urinary albumin excretion over time (*P* = .04 for between-group difference after 24 months), but no effect was observed on renal function.

Conclusion

In patients with mild to moderate chronic kidney disease, 18 months of a treatment strategy along with well-controlled blood pressure reduced CC-IMT and urinary albumin excretion and increased BA-FMD.

INTRODUCTION

Patients with mild to moderate chronic kidney disease (CKD) have an increased risk of cardiovascular disease,[1-6] which cannot fully be explained by the presence of known cardiovascular risk factors such as hypertension, diabetes, smoking, and dyslipidemia.[3, 7-9] Therefore, other atherothrombotic mechanisms play a role.[8-9] In the last few years, compelling evidence has emerged pointing to the contributing role of oxidative stress in the pathogenesis of cardiovascular complications in CKD.[10-11] Oxidative stress in patients with CKD has been attributed to the effects of uremic toxins, angiotensin II, proinflammatory cytokines, and hyperhomocysteinemia.[12-13]

Statins have been shown to reduce oxidative stress in hypercholesterolemic patients.[14-15] Vitamin E supplementation and homocysteine-lowering therapy have also been shown to reduce oxidative stress in several patient populations.[10, 16-17] However, in dialysis patients,

studies aimed at reducing cardiovascular events with statins[18] and homocysteine-lowering therapy[19] have not shown positive results. A possible explanation for these disappointing findings is that patients at the start of dialysis often have advanced cardiovascular disease,[2] which may be difficult to reverse in this phase. However, only a few cardiovascular intervention studies have been performed on patients with mild to moderate CKD,[20] and most of the large intervention trials with statins have excluded patients with moderate renal failure.

In view of these considerations, we designed the Anti-Oxidant Therapy in Chronic Renal Insufficiency (ATIC) Study to examine the effect of a treatment strategy primarily designed to achieve a stepwise reduction of oxidative stress in a population of patients with mild to moderate CKD[21] and well-controlled blood pressure. The treatment strategy consisted of pravastatin, vitamin E, and homocysteine-lowering therapy on common carotid intima-media thickness (CC-IMT) (a strong surrogate marker of cardiovascular risk in the general[22] and the dialysis[23] populations), brachial artery flow-mediated dilatation (BA-FMD) (a marker of endothelial function that can be impaired by increased oxidative stress[24-25]), estimated glomerular filtration rate (eGFR), and urinary albumin excretion. Plasma-oxidized low-density lipoprotein (oxLDL)[26] and malondialdehyde[27] were measured as oxidative stress parameters. Interventions were added to the regimen every 6 months to investigate both the effects of individual interventions and the effects of the entire strategy on the end points.

METHODS

PATIENTS

Between May 2001 and December 2002, patients with a creatinine clearance of 15 to 70 mL/min per 1.73 m^2 (0.25-1.17 mL/s per m^2) (according to the Cockcroft-Gault equation) from 7 outpatient clinics in Amsterdam, The Netherlands, were screened for eligibility for participation in the ATIC Study, a randomized, double-blind, placebo-controlled trial investigating the effects of oxidative stress–lowering treatment on vascular structure and function in nondiabetic patients with chronic renal failure who had no manifest arterial occlusive disease.

DESIGN

Participants were randomized after stratification for prior use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, creatinine clearance (between 15-39 and 40-70 mL/min per 1.73 m^2 [between 0.25-0.65 and 0.66-1.17 mL/s per m^2]), and age (between 20-49 and 50-80 years). Randomization was carried out centrally by means of a computer-generated sequence involving randomized blocks of 4, and concealed envelopes were kept by 1 hospital pharmacist. Unblinding was performed after the data analysis. After randomization, participants in the treatment group were treated with pravastatin (40 mg/d), vitamin E (α-tocopherol acetate) (300 mg/d) was added to the regimen 6 months later, and homocysteine-lowering therapy (folic acid [5 mg/d], pyridoxine hydrochloride [100 mg/d], and cyanacobalamin [1 mg/d] in 1 tablet) was added 6 months after that. Patients continued this triple therapy for another 12 months (Figure 1). Patients in the placebo group received matching placebos at the onset and 6 and 12 months later. Adherence to therapy was assessed by counting leftover pills. Subjects who were not using angiotensin-converting enzyme inhibitors or angiotensin receptor blockers at inclusion received an angiotensin-

converting enzyme inhibitor (fosinopril [10 mg/d]) for at least 2 weeks before the baseline measurements and randomization. Those who were taking angiotensin receptor blockers continued taking them. During the following visits, blood pressure was controlled according to a standard protocol in which hydrochlorothiazide (a loop diuretic was administered if the eGFR was <30 mL/min), metoprolol succinate, amlodipine mesilate, or doxazosin were added in that order to achieve a blood pressure of less than 140/90 mm Hg. Measurement of the CC-IMT and BA-FMD and laboratory tests were performed in all cases at randomization and at 6, 12, and 18 months after randomization. Laboratory tests were also performed after 24 months. We excluded individuals with diabetes mellitus (American Diabetes Association criteria), active vasculitis, nephrotic syndrome, renal transplantation, a fasting total cholesterol level higher than 270 mg/dL (7.00 mmol/L), cholesterol-lowering therapy within 3 months prior to inclusion, or ischemic coronary, cerebrovascular, or peripheral arterial disease. Ninety-three patients (out of 118 eligible patients) took part in the study (Figure 1). Written informed consent was obtained from all participants, and the study was approved by the ethical committees at each center.

PROCEDURES

Patients were examined in fasting state in a temperature-controlled (25°C) room. Data were collected with regard to age, medications, and smoking status (having smoked in the past year), and a history was obtained to exclude peripheral, cerebral, and coronary vascular disease. After 30 minutes of rest, blood pressure was measured with an oscillometric device (Press-Mate BP-8800; Colin Co, Komaki City, Japan) and expressed as the mean value of 6 measurements over a period of 30 minutes. Blood samples were collected after 15 minutes of rest and immediately placed on ice; they were centrifuged within 15 minutes and stored at –80°C until analysis.

CAROTID ARTERY ULTRASONOGRAPHY

The CC-IMT measurements were performed using a medical scanner (Scanner 350; Pie Medical, Maastricht, The Netherlands) with a linear array transducer of 7.5 MHz attached to a data registration and processing unit (Wall Track System II; Pie Medical) as described in detail elsewhere.[28-29]

BRACHIAL ARTERY ULTRASONOGRAPHY

The measurement protocol for BA-FMD has also been described in detail elsewhere.[30-31] Briefly, baseline diameter (mean of 3 measurements) and peak flow velocity (mean of 2 measurements) were determined. A pressure cuff, placed on the forearm, was then inflated and kept constant at suprasystolic pressure. After 5 minutes, the cuff was released to increase blood flow. After cuff release, maximum peak flow velocity was measured within 15 seconds and diameter was measured at 45, 90, 120, 150, and 300 seconds. The BA-FMD was calculated as the percentage of change in the maximum postocclusion diameter of the brachial artery relative to the mean baseline diameter.

REPRODUCIBILITY

All ultrasound measurements at each visit were performed by a single observer who was blinded to the treatment allocation. Reproducibility was assessed in 10 healthy subjects (43 ± 13 [mean $\pm$ SD] years) who were examined by the same observer twice, 3 weeks

apart. The intraobserver coefficient of variation (CV) (SD of the mean difference/[2 x pooled mean]$^{1/2}$) was 10% for the CC-IMT measurement and 15% for the BA-FMD measurement.

LABORATORY ANALYSES

Serum creatinine concentration was assessed by a kinetic Jaffé method. Plasma total (free plus protein-bound) homocysteine was measured with an automated fluorescence polarization immunoassay analyzer (IMx; Abbott Laboratories, Abbott Park, Ill), with an interassay CV of less than 4%.[32] Renal function was estimated by the Modification of Diet in Renal Disease (MDRD) study equation (eGFR in milliliters per minute per 1.73 m^2, per Levey equation 7)[33] and by the Cockcroft-Gault and Dubois formulas (creatinine clearance in milliliters per minute per 1.73 m^2).[34-35]

Urinary albumin was measured in a 24-hour urine collection at each visit and analyzed using a microalbumin antiserum analyzer (Beckman Array 360 Analyzer; Global Medical Instrumentation Inc, Clearwater, Minn). The CV for the between-run imprecision was 5.0% at a mean concentration of 11.4 mg/L and 5.0% at a mean concentration of 72.6 mg/L.
The plasma concentration of oxLDL was measured by a competitive enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden).[36] The intra-assay and interassay CVs were 4.8 and 7.8%, respectively. Malondialdehyde in EDTA plasma was determined after reaction with thiobarbituric acid, with an added alkaline hydrolysis step as described elsewhere.[37] The within-run and between-run variation was 3.5 and 8.7%, respectively.

POWER CALCULATION

The number of patients needed to detect an absolute CC-IMT difference of 0.09 mm between the groups over 18 months with 3 longitudinal measurements with a power of 90%, an αvalue of 0.05, and an SD of 0.13 was 38 patients per group. Taking into account a dropout percentage of 20%, the number needed per group was 47 patients.

STATISTICAL ANALYSES

Statistical analysis was performed with Stata 7.0 (Stata Corp, College Station, Tex). All analyses were performed according to the intention-to-treat principle. Outcome variables were analyzed with generalized estimating equations, an established longitudinal data analysis technique.[38] In the primary generalized estimating equations model, the outcome variable studied (eg, CC-IMT or BA-FMD) was analyzed as a dependent variable using treatment strategy (1, intervention group; 0, placebo group) as a key independent variable adjusted for time and, if appropriate, for previous observations using extra independent variables. Also, to evaluate effect modification, the product term of group and time (group x time) was added as an independent variable. In case of skewed data, analyses were performed after log transformation.

Data are presented in graphs indicating means with standard errors. All these variables except urinary albumin were normally distributed. $P<.05$ was considered statistically significant.

RESULTS

Table 1 shows the baseline characteristics of the participants. Of 93 patients who were included in the study, 6 withdrew after undergoing the baseline measurement and 87 underwent the second measurement and were included in the final analysis (Figure 1). After 2 years, 72% of the patients in the treatment group and 77% of the patients in the placebo group were still taking the drugs. Compliance at each follow-up visit was defined as consumption of at least 80% of the scheduled tablets since the previous visit. Four patients in the treatment group and 2 patients in the placebo group consumed more than 60% but less than 80% of the allocated tablets during the study period; all other participants took at least 80% of their scheduled tablets.

COMMON CAROTID INTIMA-MEDIA THICKNESS

After 18 months, the mean CC-IMT had decreased from 0.68 to 0.63 mm in the treatment group, whereas it had increased from 0.65 to 0.71 mm in the placebo group (P<.001 for between-group difference) (Figure 2). After adjustment for baseline values of CC-IMT, the treatment strategy was associated with a CC-IMT lowering of 0.13 mm (95% confidence interval [CI], 0.10-0.16 mm) at 18 months. The largest change in CC-IMT (from 0.68 to 0.65 mm in the treatment group; P<.001 for between-group difference after 6 months) was seen in the first 6 months of therapy. After adjustment for baseline values of CC-IMT, the treatment strategy was associated with a CC-IMT lowering of 0.07 mm (95% CI, 0.05-0.09 mm) after 6 months.

BRACHIAL ARTERY FLOW-MEDIATED DILATATION

After 18 months, the BA-FMD had increased from 4.66% to 7.56% in the treatment group, whereas it had decreased from 6.21% to 4.73% in the placebo group (P<.001 for between-group difference after 18 months) (Figure 3). After adjustment for baseline values of BA-FMD and time, the treatment strategy was associated with a BA-FMD increase of 3.18% (95% CI, 1.23%-5.13%) after 18 months. After 6 months, the BA-FMD had increased from 4.66% to 6.73% in the treatment group and from 6.21% to 6.43% in the placebo group (P = .11 for between-group difference after 6 months) (Figure 3).

RENAL FUNCTION

After 24 months, the mean eGFR (MDRD formula) had decreased from 35 to 33 mL/min per 1.73 m^2 (from 0.58 to 0.55 mL/s per m^2) in the placebo group and increased from 32 to 35 mL/min per 1.73 m^2 (from 0.53 to 0.58 mL/s per m^2) in the treatment group (P = .89 for between-group difference) (Figure 4). After adjustment for baseline values and time, the treatment strategy was associated with a 0.10 mL/min per 1.73 m^2 (0.002 mL/s per m^2) (95% CI, –2.11 to 1.92 mL/min per 1.73 m^2 [95% CI, –0.035 to 0.032 mL/s per m^2]) MDRD decrease at 18 months and a 0.06 mL/min per 1.73 m^2 (0.001 mL/s per m^2) (95% CI, –2.55 to 2.66 mL/min per 1.73 m^2 [95% CI, –0.042 to 0.044 mL/s per m^2]) MDRD increase at 24 months.

URINARY ALBUMIN EXCRETION

After 24 months, the median urinary albumin excretion had changed from 71 mg/24 h (range, 3-2601 mg/24 h) to 107 mg/24 h (range, 5-3545 mg/24 h) in the placebo group and from 45

mg/24 h (range, 3-3420 mg/24 h) to 77 mg/24 h (3-2509 mg/24 h) in the treatment group. After adjustment for baseline values and time, the treatment strategy was associated with a 34% (95% CI, 3%-44%; *P* = .04) reduction of urinary albumin excretion compared with the placebo group after 24 months (Figure 5). After 6 months, the median urinary albumin excretion had changed from 71 mg/24 h (range, 3-2601 mg/24 h) to 94 mg/24 h (range, 1.6-3927 mg/24 h) in the placebo group and from 45 mg/24 h (range, 3-3420 mg/24 h) to 87 mg/24 h (range, 6-2521 mg/24 h) in the treatment group.

After adjustment for baseline values and time, the treatment strategy was associated with a 20% (95% CI, 10%-33%; *P* = .02) reduction of urinary albumin excretion compared with the placebo group at 6 months. Additional analyses showed a 19% (95% CI, 5%-30%; *P* = .008) reduction in urinary albumin excretion in the treatment group in the 50 patients with urinary albumin excretion of more than 30 mg/24 h at baseline, but no effect in the 30 patients with urinary albumin excretion of less than 30 mg/24 h at baseline.

BLOOD PRESSURE

After 24 months, the mean ± SD systolic blood pressure was 134 ± 27 mm Hg in the treatment group and 134 ± 22 mm Hg in the placebo group (*P* = .14 for between-group difference). There was no statistically significant difference in the systolic blood pressure at any point during the study (Figure 6). After 24 months, the mean diastolic blood pressure was 80 ± 12 mm Hg in the treatment group and 76 ± 11 mm Hg in the placebo group (*P* = .06 for between-group difference). Adjustment for systolic, diastolic, or mean blood pressure difference at any point in the analyses of CC-IMT, BA-FMD, or urinary albumin excretion did not alter the above-mentioned results.

After 24 months, there was a strong and significant reduction of oxLDL (*P*<.001) and low-density lipoprotein cholesterol (*P*<.001) levels with the treatment strategy (Table 2). There was no significant reduction of plasma malondialdehyde (*P* = .13) during the study period (Table 2). There were 19 dropouts (11 from the treatment group and 8 from the placebo group) and 6 cardiovascular events during the study (Table 3).

COMMENT

The main finding of this study is that, in patients with mild to moderate nondiabetic CKD who had no manifest arterial occlusive disease and had well-controlled blood pressure, 18 months of treatment with an oxidative stress–lowering strategy consisting of pravastatin, vitamin E, and homocysteine-lowering therapy resulted in a statistically significant reduction in CC-IMT (*P*<.001) and a statistically significant improvement in BA-FMD (*P* = .001). There was no statistically significant effect on eGFR (*P* = .89). However, treatment was associated with an attenuated increase in urinary albumin excretion over time.

Cardiovascular morbidity and mortality are extremely high in patients with end-stage renal disease,[1-2] and the results of intervention studies aimed at the reduction of cardiovascular events with statins[18] and homocysteine-lowering therapy[19] in these patients have been disappointing. These results may suggest that the extent and nature of vascular disease in patients with end-stage renal disease makes such treatment options less effective than in other patient groups. Therefore, we evaluated whether intervention at an earlier stage of

CKD (Kidney Disease Outcomes Quality Initiative [K/DOQI] stages 2 through 4[21]) would have beneficial effects on 3 strong surrogate estimates of cardiovascular outcome,[22, 25, 39] ie, CC-IMT, BA-FMD, and urinary albumin excretion. Furthermore, and in contrast with most other lipid trials,[40-42] the design of our study included formal control of blood pressure (<140/90 mm Hg) using a strict protocol. Blood pressure control is extremely important, as hypertension is very frequent in patients with CKD, and adequate blood pressure control in patients with mild to moderate renal disease slows the decline of the eGFR and decreases cardiovascular morbidity and mortality.[43] Renin-angiotensin system blockade, in particular, has been shown to reduce proteinuria and to retard the progression of CKD, in part independent of blood pressure lowering.[43] Therefore, the results of the present study should be interpreted as the effect of the treatment strategy in conjunction with well-controlled blood pressure.

Very few data are available on the effects of statins on cardiovascular outcomes in patients with mild to moderate CKD (K/DOQI stages 2 through 4) and adequately controlled blood pressure, because patients with moderate CKD (stages 3 and 4) were usually excluded from the large cardiovascular outcome trials with statins,[44] and/or blood pressure control was not included in the design of those trials.[40-42] On the other hand, subgroup analyses of a limited number of lipid trials (Anglo-Scandinavian Cardiac Outcomes Trial[45] and the Pravastatin Pooling Project[20]) do suggest that statin treatment may reduce cardiovascular events in patients with stages 1 through 3 CKD. These data provide an indirect indication of the beneficial effects of lipid lowering in stages 1 to 3 CKD. It is important to realize, however, that patients with stage 4 CKD (eGFR, 15-29 mL/min per 1.73 m^2) were absent or the numbers were too small for analysis in these trials. In a recent study, Isbel *et al*[46] showed that, when compared with usual care, a multiple risk factor intervention program in a population of patients with stages 4 and 5 CKD was not associated with reduction in CC-IMT or with improvement in endothelial function. However, only 25% of the patients in Isbel and colleagues' study had stage 4 CKD, and those patients were not analyzed separately.

Therefore, to our knowledge, the ATIC Study is the first randomized, placebo-controlled trial examining the effect of an oxidative stress–lowering strategy in a population of patients with mild to moderate nondiabetic stages 3 and 4 CKD without manifest cardiovascular disease. Also, and in contrast to the above-mentioned studies, 45% (42/93) of our patient population had K/DOQI stage 4 CKD, equally divided between the treatment and the placebo groups (22 patients in the treatment group and 20 patients in the placebo group). Most of the remaining patients (48/93) had K/DOQI stage 3 CKD (25 patients in the treatment group and 23 patients in the placebo group). The treatment strategy had beneficial effects on the CC-IMT and BA-FMD in patients with stages 3 and 4 CKD (data not shown).

The systolic blood pressure did not differ statistically significantly (P = .14) between the 2 groups at any point, and adjustment for systolic, diastolic, or mean blood pressure difference did not alter the CC-IMT, BA-FMD, or urinary albumin excretion results. Therefore, according to our study findings, we conclude that the treatment strategy described herein in conjunction with adequately controlled blood pressure has beneficial effects for patients with stage 3 or 4 CKD who have no prior cardiovascular disease.

A few studies have demonstrated a renoprotective effect of statins.[47-49] However, most of these studies were short term, with small patient populations, or subgroup analyses from large statin trials involving subjects at high risk for cardiovascular events but with mild CKD or normal renal function at baseline. After 2 years of treatment in our patient population, we could not demonstrate a statistically significant effect on the eGFR between the groups. Our study was not powered, and the follow-up period may have been too short to demonstrate any effect on the eGFR.

However, we were able to demonstrate a significant attenuation of the increase in urinary albumin excretion over time in the treatment group. Additional analyses suggested that these effects were limited to individuals with urinary albumin excretion of more than 30 mg/24 h, but the hazards of such analyses are well known, and, clearly, this result requires confirmation. Urinary albumin excretion may be a marker of endothelial dysfunction[50] and oxLDL is known to down-regulate endothelial nitric oxide activity.[51] Therefore, a reduction in oxLDL may have contributed to the improvement in endothelial function, as shown in our population (Figure 3), and thereby to the reduction of urinary albumin excretion.

We studied a selected population of patients with mild to moderate CKD. Our study had limited power and was too short to detect an effect on clinical cardiovascular end points. In other populations, large trials with vitamin E and homocysteine lowering have not shown any beneficial effects on cardiovascular events. However, during the design period of our study, the then-available information suggested that these vitamins could have beneficial effects in patients with renal failure because these patients were known to have increased oxidative stress. Also, small studies with vitamin E in dialysis patients[52] at that time showed some promising results. We decided to use the treatment strategies comcomitantly to reduce the number of patients needed to perform this study and to achieve a maximum oxidative stress reduction in the treatment group. Furthermore, we decided to add interventions sequentially and planned to evaluate the effects of individual treatments. We expected (in retrospect, wrongly) the maximum effect of each intervention to be achieved within 6 months after the given intervention and/or that the additional effect of the next step would be clearly distinguishable from the effects of the previous step. Decreases in CC-IMT and improvement in BA-FMD were observed during the whole study period (Figures 2 and 3). In retrospect, we are unable to draw any conclusions on the individual effects of these interventions. Also, the treatment modalities of the present study certainly have effects independent of oxidative stress lowering. We therefore cannot draw any conclusions as to whether the observed improvements were the results of the oxidative stress lowering or of other effects such as reduction of lipid levels.

In conclusion, in nondiabetic patients with mild to moderate CKD with adequately controlled blood pressure and without clinical features of atherosclerosis, a treatment strategy consisting of pravastatin, vitamin E, and homocysteine-lowering therapy resulted in a significant reduction in CC-IMT and a significant improvement in endothelial function and urinary albumin excretion. No significant effect on eGFR was seen. These results suggest, but do not prove, that this treatment strategy might safely reduce the burden of cardiovascular events in this population. Thus, larger studies carried out over a long period with clinical end points will be required to confirm and validate these results.

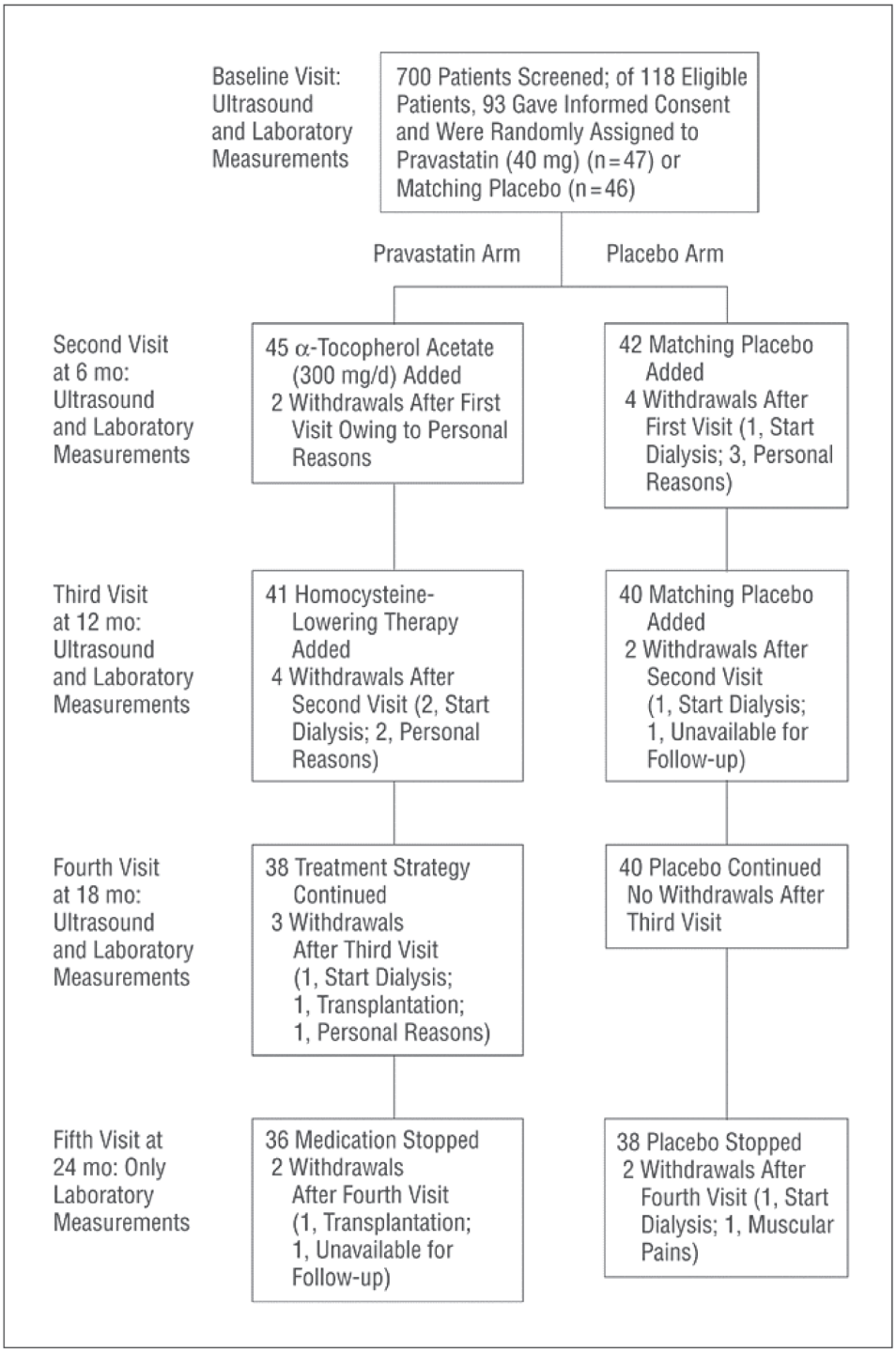

Figure 1. Flow of participants through each stage of the study.

Table 1. Baseline Characteristics of the Participants*

Variable	Placebo Group (n = 46)	Treatment Group (n = 47)	*P* Value
Male, No. (%)	29 (63)	24 (51)	.25
Age, y	52 ± 13	54 ± 11	.53
BMI	26 ± 4	27 ± 5	.47
Smokers, No. (%)	17 (37)	16 (34)	
Blood pressure, mm Hg			
Systolic	134 ± 22	136 ± 20	.63
Diastolic	78 ± 13	79 ± 11	.73
Mean	97 ± 15	98 ± 13	.67
Pulse	56 ± 13	57 ± 13	.64
Lipids, mmol/L			
Total cholesterol	5.4 ± 1.0	5.8 ± 1.5	.16
LDL cholesterol	3.3 ± 0.9	3.8 ± 0.9	.008
HDL cholesterol	1.3 ± 0.4	1.2 ± 0.3	.23
Triglycerides	1.9 ± 1.1	1.8 ± 1.0	.80
Plasma homocysteine, µmol/L	22.5 ± 11.3	20.2 ± 6.8	.26
Oxidative stress parameters			
oxLDL, U/L	61 ± 16	68 ± 12	.02
Plasma malondialdehyde, µmol/L	8.07 ± 1.5	7.95 ± 1.3	.66
Renal function			
MDRD formula, mL/s per m^2	0.58 ± 0.23	0.53 ± 0.22	.38
Serum creatinine, µmol/L	199 ± 70	211 ± 96	.46
Cockcroft-Gault formula, mL/s per m^2	0.65 ± 0.25	0.63 ± 0.27	.68
Urinary albumin excretion, median (range), mg/24 h	71 (3-2601)	45 (3-3420)	.56
CC-IMT, mm	0.65 ± 0.12	0.68 ± 0.15	.17
BA-FMD, %	6.21 ± 5.16	4.66 ± 3.19	.20
Antihypertensive medications, No.			
ACE inhibitors	28	33	
Angiotensin receptor blockers	10	9	
Diuretics	18	27	
β-Blockers	19	15	
α-Blockers	3	2	
Calcium channel blockers	8	13	
Underlying renal diseases, No. (%)			
Hypertension	17 (37)	12 (26)	
Polycystic kidney disease	9 (20)	4 (8)	

Abbreviations: ACE, angiotensin-converting enzyme; BA-FMD, brachial artery flow-mediated vasodilatation; BMI, body mass index (weight in kilograms divided by height in meters squared); CC-IMT, common carotid intima-media thickness; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDRD: Modification of Diet in Renal Disease; oxLDL, oxidized LDL.

Conventional unit conversion factors: to convert total, LDL, and HDL cholesterol and triglycerides to milligrams per deciliter, divide by 0.0259 and 0.0113, respectively; to convert serum creatinine to milligrams per deciliter, divide by 88.4; to convert plasma homocysteine and malondialdehyde to milligrams per liter, divide by 7.397 and 138.9, respectively; to convert MDRD and Cockcroft-Gault formulas to milliliters per minute per 1.73 m^2, divide by 0.0167.

*Values are expressed as mean ± SD unless indicated otherwise.

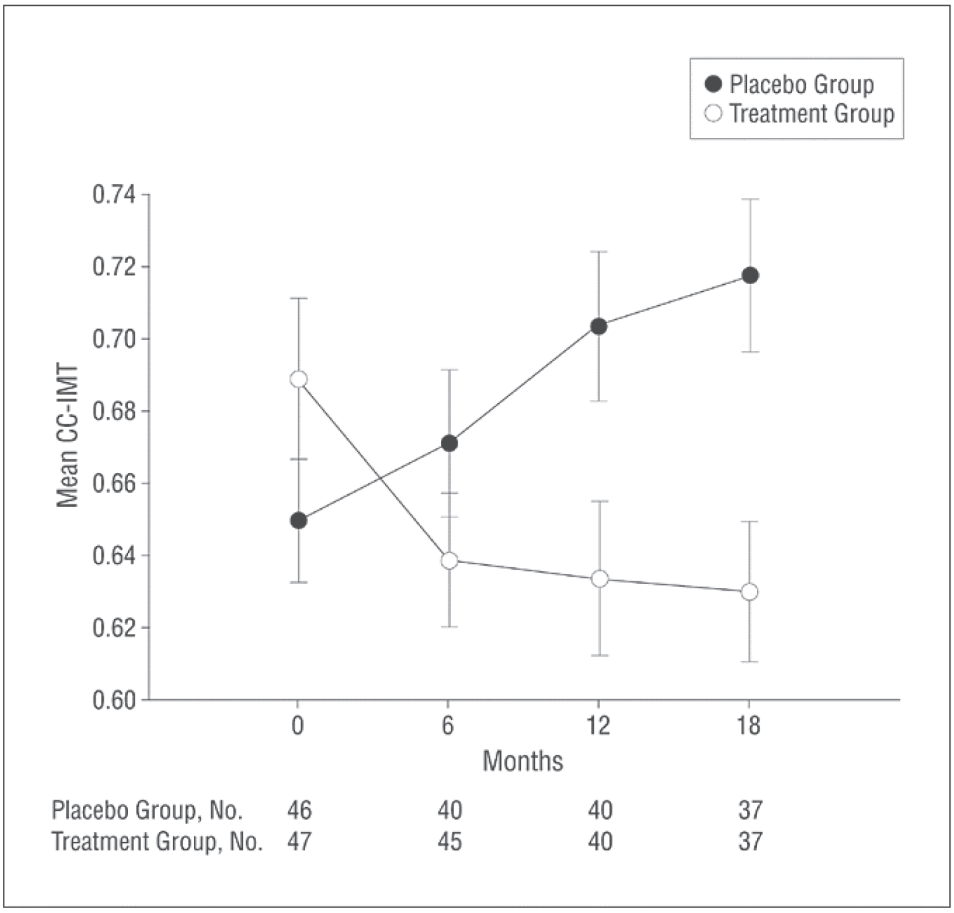

Figure 2. Change in mean (SE) common carotid intima-media thickness (CC-IMT), with the *P* values for between-group differences. For 0 to 6 months, 6 to 12 months, 12 to 18 months, and 0 to 18 months, *P*<.001. Error bars indicate SE.

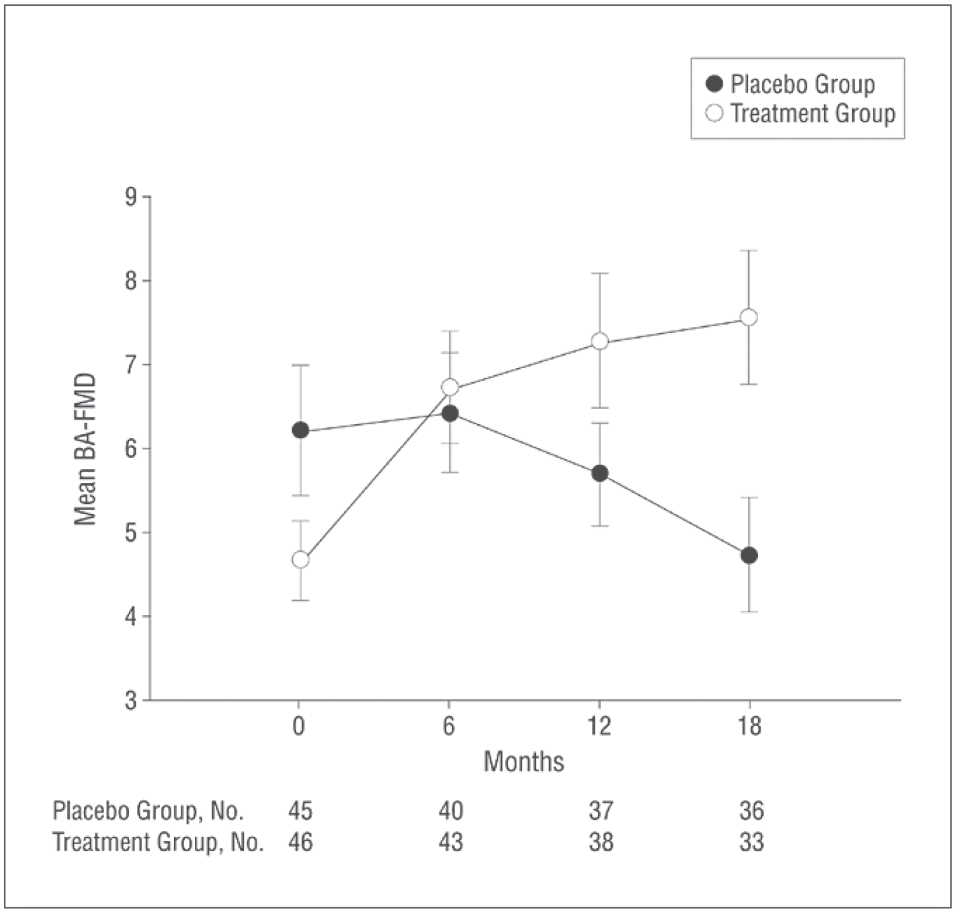

Figure 3. Change in mean (SE) brachial artery flow-mediated vasodilatation (BA-FMD), with the *P* values for between-group differences. For 0 to 6 months, *P* = 11; for 6 to 12 months, *P* = .009; and for 12 to 18 months and 0 to 18 months, *P* = .001. Error bars indicate SE.

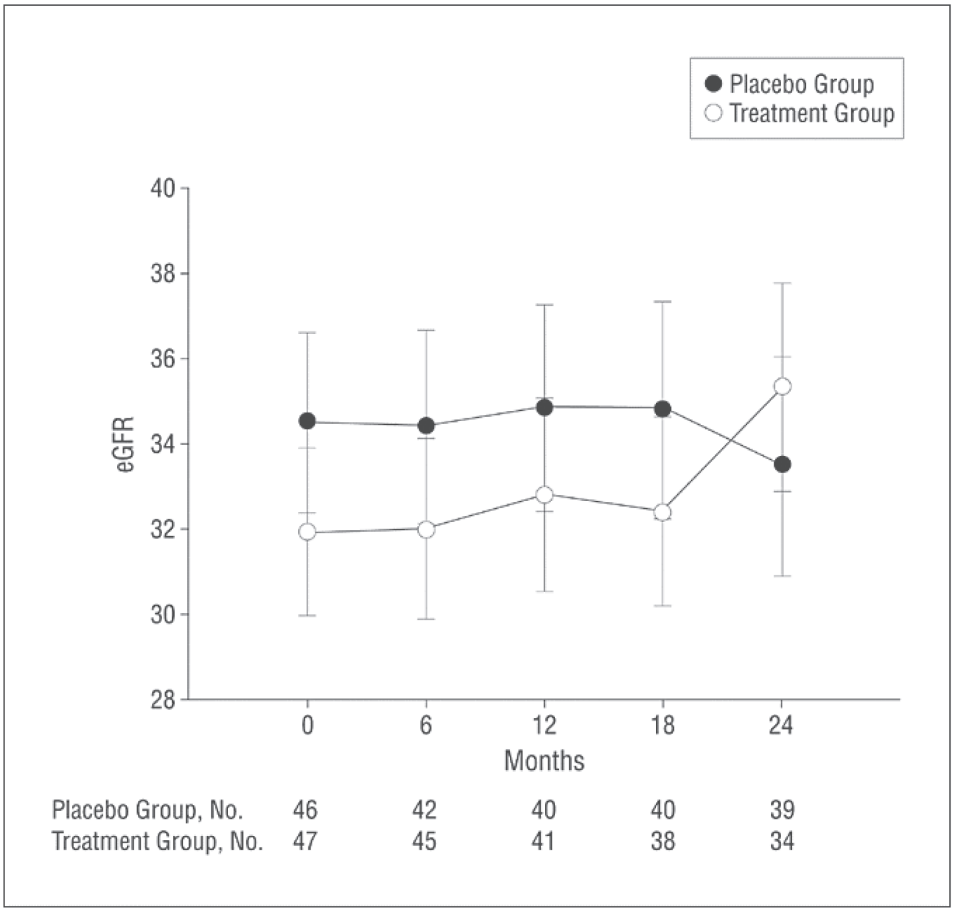

Figure 4. Change in mean (SE) estimated glomerular filtration rate (eGFR) based on the Modification of Diet in Renal Disease formula (milliliters per minutes per 173 m^2, per Levey equation 7), with the *P* values for between-group differences. For 0 to 6 months, *P* = .79; for 6 to 12 months, *P* = .60; for 12 to 18 months, *P* = .21; for 18 to 24 months, *P* = .39; and for 0 to 24 months, *P* = .33. Error bars indicate SE.

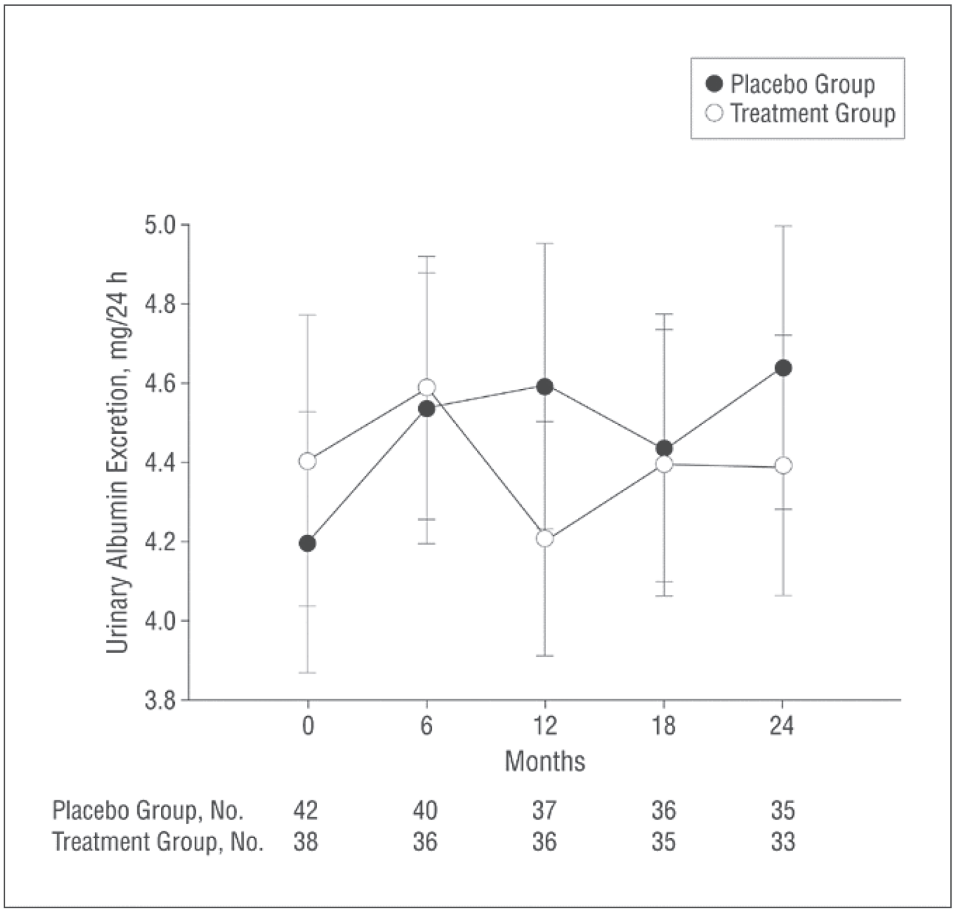

Figure 5. Change in mean (SE) log-transformed urinary albumin excretion, with the *P* values for between-group differences. For 0 to 6 months, *P* = .03; for 6 to 12 months, *P* = .02; for 12 to 18 months, *P* = .03; for 18 to 24 months, *P* = .06; and for 0 to 24 months, *P* = .04. Error bars indicate SE.

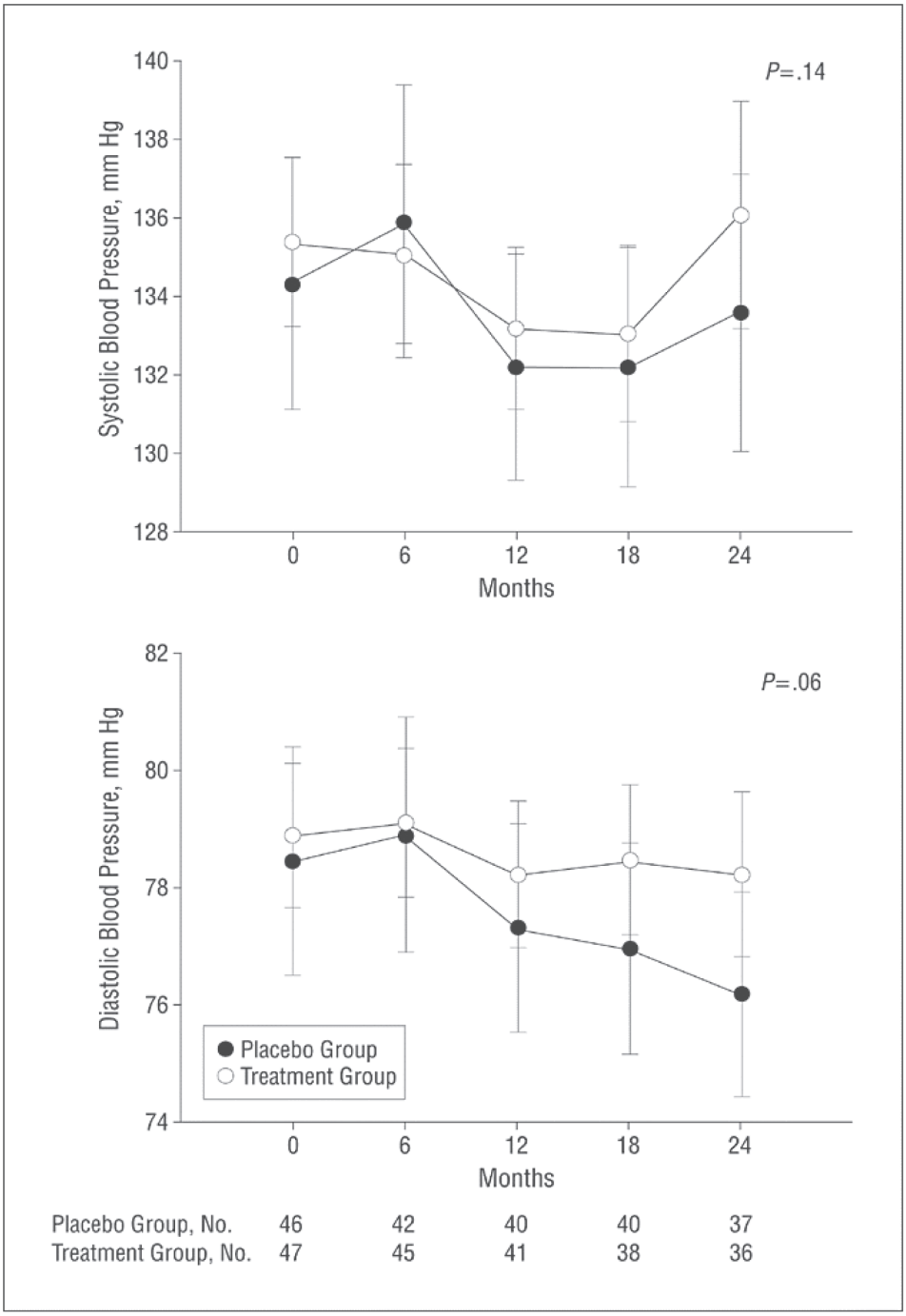

Figure 6. Change in mean (SE) blood pressure, with the *P* values for between-group differences. Error bars indicate SE.

Table 2. Changes in Oxidized Low-Density Lipoprotein (LDL) Cholesterol and Plasma Malondialdehyde, LDL Cholesterol, High-Density Lipoprotein (HDL) Cholesterol, Triglyceride, and Homocysteine Levels During the Study*

Variable	At Baseline	At 6 mo	At 12 mo	At 18 mo	At 24 mo	*P* Value for Between-Group Differences During Entire Study Period
Mean oxidized LDL cholesterol, U/L						<.001
Treatment group	67.48 ± 12.35	56.67 ± 13.49	55.97 ± 12.25	56.33 ± 14.19	57.31 ± 13.87	
Placebo group	60.57 ± 15.59	64.61 ± 15.07	63.19 ± 15.67	62.11 ± 15.19	62.13 ± 14.57	
Mean plasma malondialdehyde, µmol/L						.13
Treatment group	7.95 ± 1.29	7.66 ± 1.14	7.88 ± 1.09	8.08 ± 1.06	8.18 ± 1.45	
Placebo group	8.07 ± 1.51	8.16 ± 1.52	8.24 ± 1.67	8.34 ± 1.67	8.25 ± 1.79	
Mean plasma LDL cholesterol, mmol/L						<.001
Treatment group	3.79 ± 0.93	2.65 ± 0.85	2.67 ± 0.76	2.74 ± 0.81	2.80 ± 0.77	
Placebo group	3.27 ± 0.88	3.38 ± 0.95	3.36 ± 0.92	3.39 ± 0.93	3.41 ± 1.07	
Mean plasma HDL cholesterol, mmol/L						.63
Treatment group	1.21 ± 0.35	1.33 ± 0.40	1.31 ± 0.43	1.34 ± 0.44	1.43 ± 0.45	
Placebo group	1.30 ± 0.42	1.26 ± 0.38	1.30 ± 0.34	1.33 ± 0.39	1.37 ± 0.41	
Mean plasma triglycerides, mmol/L						.06
Treatment group	1.84 ± 1.07	1.60 ± 0.97	1.82 ± 1.77	1.94 ± 1.96	1.74 ± 1.29	
Placebo group	1.90 ± 1.10	2.02 ± 1.19	2.14 ± 1.51	1.84 ± 0.94	1.66 ± 0.82	
Mean plasma homocysteine, µmol/L						.001
Treatment group	20.16 ± 6.80	19.76 ± 6.46	17.31 ± 6.02	11.31 ± 4.66	10.45 ± 4.02	
Placebo group	22.45 ± 11.27	21.87 ± 9.91	18.71 ± 8.63	19.68 ± 10.44	20.22 ± 12.06	

Conventional unit conversion factors: to convert plasma malondialdehyde to milligrams per liter, divide by 138.9; to convert LDL and HDL cholesterol and triglycerides to milligrams per deciliter, divide by 0.0259 and 0.0113, respectively; to convert homocysteine to milligrams per liter, divide by 7.397.
*Values other than *P* values are expressed as mean ± SD.

Table 2. Changes in Oxidized Low-Density Lipoprotein (LDL) Cholesterol and Plasma Malondialdehyde, LDL Cholesterol, High-Density Lipoprotein (HDL) Cholesterol, Triglyceride, and Homocysteine Levels During the Study*

Table 3. Clinical Cardiovascular Events and Adverse Events and Withdrawals in Both Groups During the Study

Variable	Treatment Group	Placebo Group
Cardiovascular events, No.	2 (2 myocardial infractions)	6 (3 angina pectoris, 1 myocardial infarction, 1 transient ischemic attack, and 1 sudden cardiac death)
Dropouts, No.	11 (5 for personal reasons, 1 unavailable for follow-up, and 5 developed end-stage renal disease)	8 (1 for muscular pains, 3 for personal reasons, 1 unavailable for follow-up, and 3 developed end-stage renal disease)
Addition of statins by the treating physician	None	None

Table 3. Clinical Cardiovascular Events and Adverse Events and Withdrawals in Both Groups During the Study

Reference List

1. Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. *J Am Soc Nephrol.* 1998;9(suppl):S16-S23.

2. Baigent C, Burbury K, Wheeler D. Premature cardiovascular disease in chronic renal failure. *Lancet.* 2000;356:147-152.

3. Henry RM, Kostense PJ, Bos G; et al. Mild renal insufficiency is associated with increased cardiovascular mortality: The Hoorn Study. *Kidney Int.* 2002;62:1402-1407.

4. Muntner P, He J, Hamm L, Loria C, Whelton PK. Renal insufficiency and subsequent death resulting from cardiovascular disease in the United States. *J Am Soc Nephrol.* 2002;13:745-753.

5. Drey N, Roderick P, Mullee M, Rogerson M. A population-based study of the incidence and outcomes of diagnosed chronic kidney disease. *Am J Kidney Dis.* 2003;42:677-684

6. Vanholder R, Massy Z, Argiles A; et al. Chronic kidney disease as cause of cardiovascular morbidity and mortality. *Nephrol Dial Transplant.* 2005;20:1048-1056.

7. Cheung AK, Sarnak MJ, Yan G; et al. Atherosclerotic cardiovascular disease risks in chronic hemodialysis patients. *Kidney Int.* 2000;58:353-362.

8. Stenvinkel P, Pecoits-Filho R, Lindholm B. Coronary artery disease in end-stage renal disease: no longer a simple plumbing problem. *J Am Soc Nephrol.* 2003;14:1927-1939.

9. Zoccali C. Cardiovascular risk in uremic patients—is it fully explained by classical risk factors? *Nephrol Dial Transplant.* 2000;15:454-457.

10. Himmelfarb J, Stanvinkel P, Ikizler TA, Hakim RM. The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney Int.* 2002;62:1524-1538.

11. Terawaki H, Yoshimura K, Hasegawa T; et al. Oxidative stress in enhanced in correlation with renal dysfunction: examination with the redox state of albumin. *Kidney Int.* 2004;66:1988-1993.

12. Vaziri ND. Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. *Curr Opin Nephrol Hypertens.* 2004;13:93-99.

13. Heinecke JW, Rosen H, Suzuki LA, Chait A. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J Biol Chem.* 1987;262:10098-10103.

14. Sakabe K, Fukuda N, Wakayama K, Nada T, Shinohara H, Tamura Y. Lipid-altering changes and pleiotropic effects of atorvastatin in patients with hypercholesterolemia. *Am J Cardiol.* 2004;94:497-500.

15. Zhang B, Noda K, Matsunaga A, Kumagai K, Saku K. A comparative crossover study of the effects of fluvastatin and pravastatin (FP-COS) on circulating autoantibodies to oxidized LDL in patients with hypercholesterolemia. *J Atheroscler Thromb.* 2005;12:41-47.

16. Ullegaddi R, Powers HJ, Gariballa SE. B-group vitamin supplementation mitigates oxidative damage after acute ischemic stroke. *Clin Sci (Lond).* 2004;107:477-484.

17. Harris A, Devaraj S, Jialal I. Oxidative stress, alpha-tocopherol therapy, and atherosclerosis. *Curr Atheroscler Rep.* 2002;4:373-380.

18. Wanner C, Krane V, Marz W; et al. Atorvastatin in patients with type 2 diabetes mellitus undergoing hemodialysis. *N Engl J Med.* 2005;353:238-248.

19. Wrone EM, Hornberger JM, Zehnder JL, McCann LM, Coplon NS, Fortmann SP. Randomized trial of folic acid for prevention of cardiovascular events in end-stage renal disease. *J Am Soc Nephrol.* 2004;15:420-426.

20. Tonelli M, Isles C, Curhan GC; et al. Effect of pravastatin on cardiovascular events in people with chronic kidney disease. *Circulation.* 2004;110:1557-1563.

21. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification and stratification. *Am J Kidney Dis.* 2002;39(suppl 1):S1-S266.

22. Hodis HN, Mack WJ, LaBree L; et al. The role of carotid arterial intima-media thickness in predicting clinical coronary events. *Ann Intern Med.* 1998;128:262-269.

23. Benedetto FA, Mallamaci F, Tripepi G, Zoccali C. Prognostic value of ultrasonographic measurement of carotid intima media thickness in dialysis patients. *J Am Soc Nephrol.* 2001;12:2458-2464.

24. Hasdan G, Benchetrit S, Rashid G, Green J, Bernheim J, Rathaus M. Endothelial dysfunction and hypertension in 5/6 nephrectomized rats are mediated by vascular superoxide. *Kidney Int.* 2002;61:586-590.

25. Moens AL, Goovaerts I, Claeys MJ, Vrints CJ. Flow-mediated vasodilatation: a diagnostic instrument or an experimental tool. *Chest.* 2005;127:2254-2263.

26. Holvoet P. Oxidized LDL and coronary heart disease. *Acta Cardiol.* 2004;59:479-484.

27. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis.* 2005;15:316-328.

28. Henry RMA, Kostense PJ, Spijkerman AMW; et al. Arterial stiffness increases with deteriorating glucose tolerance status: The Hoorn study. *Circulation.* 2003;107:2089-2095.

29. Nanayakkara PWB, Teerlink T, Stehouwer CDA; et al. Plasma asymmetric dimethylarginine (ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration in patients with mild to moderate renal failure. *Kidney Int.* 2005;68:2230-2236.

30. Henry RM, Ferreira I, Kostense PJ; et al. Type 2 diabetes is associated with impaired endothelium-dependent, flow-mediated dilatation, but impaired glucose metabolism is not: The Hoorn Study. *Atherosclerosis.* 2004;174:49-56.

31. Lambert J, Aarsen M, Donker AJ, Stehouwer CDA. Endothelium-dependent and -independent vasodilatation of large arteries in normoalbuminuric insulin-dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol.* 1996;16:705-711.

32. Shipchandler MT, Moore EG. Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyser. *Clin Chem.* 1995;41:991-994.

33. Levey AS, Bosch JP, Lewis BJ; et al. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation: Modification of Diet in Renal Disease Study Group. *Ann Intern Med.* 1999;130:461-470.

34. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron.* 1976;16:31-41.

35. Du Bois D, Du Bois EF. A formula to estimate the approximate surface area if height and weight be known: 1916. *Nutrition.* 1989;5:303-311.

36. Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation.* 1998;98:1487-1494.

37. van de Kerkhof J, Schalkwijk CG, Konings CJ; et al. Nepsilon-(carboxymethyl)lysine, Nepsilon-(carboxyethyl)lysine and vascular cell adhesion molecule-1 (VCAM-1) in relation to peritoneal glucose prescription and residual renal function; a study in peritoneal dialysis patients. *Nephrol Dial Transplant.* 2004;19:910-916.

38. Twisk JW. Longitudinal data analysis: a comparison between generalized estimating equations and random coefficient and random coefficient analysis. *Eur J Epidemiol.* 2004;19:769-776.

39. Hillege HL, Fidler V, Diercks GFH; et al, Prevention of Renal and Vascular End Stage Disease (PREVEND) Study Group. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. *Circulation.* 2002;106:1777-1782.

40. Shepherd J, Cobbe SM, Ford I; et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. *N Engl J Med.* 1995;333:1301-1307.

41. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. *N Engl J Med.* 1998;339:1349-1357.

42. Sacks FM, Pfeffer MA, Moye LA; et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. *N Engl J Med.* 1996;335:1001-1009.

43. Brenner BM. Retarding the progression of renal disease. *Kidney Int.* 2003;64:370-378.

44. The Scandinavian Simvastatin Survival Study (4S). Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease. *Lancet.* 1994;344:1383-1389.

45. Sever PS, Dahlof B, Poulter NR; et al. Prevention of coronary and stroke events with atorvastatin in hypertensives who have average or lower-than-average cholesterol concentrations, in the Scandinavian Cardiac Outcome Trial-Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet.* 2003;361:1149-1158.

46. Isbel NM, Haluska B, Johnson DW, Beller E, Hawley C, Marwick TH. Increased targeting of cardiovascular risk factors in patients with chronic kidney disease does not improve atheroma burden or cardiovascular function. *Am Heart J.* 2006;151:745-753.

47. Athyros VG, Mikhailidis DP, Papageorgiou AA; et al. The effect of statins versus untreated dyslipidaemia on renal function in patients with coronary heart disease: a subgroup analysis of the Greek atorvastatin and coronary heart disease evaluation (GREACE) study. *J Clin Pathol.* 2004;57:728-734.

48. Tonelli M, Moye L, Sacks FM, Cole T, Curhan GC. Effect of pravastatin on loss of renal function in people with moderate chronic renal insufficiency and cardiovascular disease. *J Am Soc Nephrol.* 2003;14:1605-1613.

49. Agarwal R. Effects of statins on renal function. *Am J Cardiol.* 2006;97:748-755.

50. Stehouwer CDA, Smulders YM. Microalbuminuria and risk for cardiovascular disease: analysis of potential mechanisms. *J Am Soc Nephrol.* 2006;17:2106-2111.

51. Hernández-Perera O, Pérez-Sala D, Navarro-Antolin J; et al. Effects of the 3-hydroxy-3-methylglutaryl-COA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J Clin Invest.* 1998;101:2711-2719.

52. Boaz M, Smetana S, Weinstein T; et al. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *Lancet.* 2000;356:1213-1218.

Chapter 7

Randomized placebo-controlled trial assessing a treatment strategy consisting of pravastatin, vitamin E, and homocysteine lowering on plasma asymmetric dimethylarginine concentration in mild to moderate CKD

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Abstract

Background: Chronic kidney disease (CKD) is associated with an increased incidence of cardiovascular disease (CVD). The Anti-oxidant Therapy In Chronic renal insufficiency study (ATIC- study) showed that a multi-step treatment strategy improved carotid intima-media thickness, endothelial function and microalbuminuria in patients with stage 2 to 4 chronic kidney disease. An increased plasma concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, has been linked to higher CVD risk in CKD patients. The aim of this study was to assess the effects of the multi-step intervention on plasma ADMA concentrations in the ATIC-study.

Study design: Secondary analysis of a randomized, double-blind, placebo-controlled trial.

Setting and participants: Ninety-three patients with creatinine clearance of 15-70 mL / min per 1.73m2 (according to the Cockcroft-Gault equation) from 7 outpatient clinics in Amsterdam, The Netherlands.

Intervention: The treatment group received sequential treatment consisting of pravastatin 40 mg/day to which, after 6 months, vitamin E 300 mg/day and after another 6 months homocysteine lowering therapy (folic acid 5 mg/day, pyridoxine 100 mg/day and vitamin B12 1 mg/day all in one tablet) were added, and were continued for another year. The control group received matching placebos.

Outcome and Measures: Plasma ADMA levels

Results: In total, 36 subjects (77%) in the treatment group and 38 subjects (83%) in the placebo group completed the study. The mean (SD) ADMA and SDMA concentration in the total study population were 0.53 ± 0.07 μmol/L and 1.14 ± 0.46 μmol/L respectively. After 24 months, there was no overall effect of the treatment strategy on ADMA concentrations (ß= -0.006, P=0.27). Analysis of separate treatment effects suggested that vitamin E significantly lowered ADMA by 4% in the treatment group compared to the placebo group (multiple adjusted P-value = 0.02).

Limitations: Study was a secondary analysis, power calculation was based on primary end point carotid intima media-thickness, mean plasma ADMA levels were relatively low,

Conclusion: Overall, a multi-step treatment strategy consisting of pravastatin, vitamin E and B vitamins had no effect on plasma ADMA levels in a stage 2-4 CKD population. This suggests that the beneficial effects of the intervention were not mediated by changes in ADMA. Possible ADMA-lowering effects of vitamin E deserve further attention.

Introduction

Chronic kidney disease (CKD) is associated with an increased incidence of cardiovascular disease (CVD) which can not be fully explained by the traditional cardiovascular risk factors.[1] Therefore, other atherothrombotic mechanisms seem to play a role. Plasma asymmetric dimethylarginine (ADMA) is a methylated product of the amino acid L-arginine in

proteins. ADMA is known to inhibit endothelium-dependant nitric oxide mediated vascular response and endothelium dependent NO bioavailability.[2] A high ADMA concentration is associated with an increased CVD in CKD patients.[3] In addition, high concentrations of ADMA are associated with an increased intima media thickness (IMT) of the carotid artery, a validated surrogate marker for atherosclerosis.[4-7] Hyperhomocysteinemia is prevalent in CKD patients and is thought to inhibit the metabolic breakdown of ADMA by impairing the activity of the enzyme dimethylarginine dimethylaminohydrolase (DDAH).[8-10] DDAH activity can also be inhibited by inflammation and oxidative stress, which are both highly prevalent in advanced CKD.[9;11]

Pharmacological interventions aimed at reducing plasma ADMA levels have shown inconsistent results. One small study demonstrated a decrease in ADMA after folate treatment in hyperhomocysteinemic subjects.[12] However, several other studies found no effect of B-vitamin treatment on ADMA in subjects with traditional CVD risk factors.[13-15] In a small study with only 8 CKD patients and 6 controls, a significant decrease in ADMA after 8 weeks of treatment with vitamin E (á-tocopherol) was found.[16] However, to our knowledge, no studies have been performed to confirm these results.

The effect of statins on ADMA levels in CKD patients is unclear. Lu *et al*. demonstrated that in a population of hypercholesterolemia patients treatment with 10 mg of rosuvasatin for 6 weeks resulted in a significant reduction in plasma ADMA levels, while Valkonen *et al*. reported no significant effect of high dose simvastatin and atorvastatin on plasma ADMA levels in hypercholesterolaemic patients.[17;18] In a very recent study, Panichi *et al* demonstrated that treatment with simvastatin 40 mg during 6 months did not lead to a reduction in plasma ADMA levels in 35 patients with CKD[19].

The current study is a secondary analysis of Anti-oxidant Therapy In Chronic renal insufficiency study (ATIC- study), which assessed the effects of a multi-step treatment strategy consisting of pravastatin, vitamin E and homocysteine lowering therapy (folic acid, vitamin B6 and vitamin B12) in patients with stage 2 to 4 CKD. In the primary analyses of this study, the intervention strategy improved carotid intima-media thickness, endothelial function and microalbuminuria.[20] We hypothesized that these beneficial effects might, at least partly, be explained by a reduction in ADMA. Our first aim was thus to assess whether the multistep intervention strategy lowered plasma ADMA levels. Because both elevated homocysteine concentrations and oxidative stress are known to inhibit DDAH activity and thereby increase ADMA levels, we also investigated the effect of treatment in subgroups stratified for baseline homocysteine and oxidized-LDL concentrations.

Materials and methods

Anti-oxidant Therapy In Chronic renal insufficiency study (ATIC- study) was a randomized, double blind, placebo-controlled clinical trial to investigate the effect of oxidative stress-reduction on vascular structure and function in patients with CKD.[20] Between May 2001 and December 2002, CKD patients (n=700) with a creatinine clearance of 15-70 mL / min per 1.73m^2 (0.25 – 1.17 mL/s per m^2) (according to the Cockcroft-Gault equation)[21] from 7 outpatient clinics in Amsterdam, The Netherlands were screened for eligibility for participation.

Patients with diabetes mellitus, active vasculitis, nephrotic syndrome, renal transplantation, hypercholesterolemia (>7.0 mmol/l), cholesterol-lowering therapy within the past 3 months or history of CVD were excluded. Out of 118 eligible patients, 93 gave informed

consent (figure 1). Participants were randomized to active treatment or placebo after stratification for prior use of angiotensin converting enzyme inhibitors (ACE-inhibitors) or angiotensin receptor blockers (ARBs), creatinine clearance (between 15-39 and 40-70 ml / min per 1.73 m2) [between 0.25 -0.65 and 0.66-1.17 mL/s per m^2] and age (between 20-49 and 50-80). Randomization was carried out centrally by means of a computer generated sequence involving randomized blocks of four and concealed envelops were kept by one central hospital pharmacist. Unblinding was performed after the primary data analysis was completed. After randomization, participants in the active treatment group were treated with pravastatin 40 mg/day for six months; subsequently vitamin E 300 mg/day (450 IU α-tocopherolacetate) was added for another six months; and lastly homocysteine-lowering therapy (folic acid 5 mg/day, pyridoxine 100 mg/day and vitamin B12 1 mg/day all in one tablet) was added for another 12 months (figure 1). The total duration of the study was 24 months. Patients in the placebo group received matching placebos at the onset, 6 and 12 months later. Adherence to therapy was assessed by returned tablet counts at each visit. Compliance at each follow-up visit was defined as consumption of at least 80% of the scheduled tablets since the previous visit. Subjects not using ACE-inhibitors or ARBs at inclusion received an ACE-inhibitor (fosinopril up to 20 mg/day titrated to achieve a blood pressure of <140/90 mmHg) for at least two weeks before the baseline measurement and randomization. Of the subjects who used ACE-inhibitors, 71%(20/28 subjects) in the intervention group and 66% (22/33 subjects) in the placebo group used fosinopril 20mg/day; the remainder of the subjects used a lower dose. Those who were on ARBs continued their ARBs. Off-label use of statins or multivitamins were not allowed and there were no dietary restrictions during the study period. Folic acid fortifications of cereals were not used widely in the Netherlands during the study period.

Measurements

Firstly, data were collected with regard to medication and smoking status (having smoked in the past year), and a detailed history was obtained to exclude clinically relevant peripheral, cerebral and coronary vascular disease at baseline. Thereafter, height and weight were measured with the individuals wearing light clothing. At baseline and during each visit (every 6 months) laboratory measurements were performed and all patients were examined in the fasting state in a supine position in a temperature-controlled (25ºC) room.

Plasma concentrations of arginine, ADMA and symmetric dimethylarginine (SDMA) were determined simultaneously by high-performance liquid chromatography as described previously[22] using modified chromatographic conditions (SDMA the biologically inactive stereoisomer of ADMA was measured because SDMA is also increased in CKD patients but not metabolized by DDAH).[1]

In short, sample clean-up was performed by solid-phase extraction on polymeric cation-exchange extraction columns using monomethylarginine as internal standard. After derivatization with ortho-phthaldialdehyde reagent containing 3-mercaptoproprionic acid, analytes were separated by isocratic reversed-phase high-performance liquid chromatography with fluorescence detection. The intra-assay coefficients of variation (CV) was < 2% for all compounds and inter-assay CVs were 3% for arginine and ADMA, and 4% for SDMA. To minimize analytical variation, all samples were analyzed at the end of the study and samples of each individual patient were analyzed in the same analytical series. Plasma concentrations of CRP were also determined because of its probable negative influence on

DDAH activity[7] and were measured with a highly sensitive in-house enzyme-linked immunosorbent assay (ELISA) with rabbit anti-CRP (Dako, Copenhagen, Denmark) as a capturing and tagging antibody, with intra- and interassay coefficients of variation of 3.8 and 4.7%, respectively. Plasma total (free plus protein-bound) homocysteine was measured with an automated fluorescence polarization immunoassay on an Abbott IMx analyzer (Abbott Laboratories, Abbott Park, IL, USA), with an interassay coefficient of variation < 4%.[23] Serum creatinine concentration was assessed by kinetic Jaffé method. Renal function was estimated by the 6-variable Modification of Diet in Renal Disease (MDRD) study equation (estimated glomerular filtration rate (eGFR) in ml/min/1.73 m^2, per Levey equation 7).[24]

The plasma concentration of oxidized LDL (oxLDL) was measured by a competitive ELISA (Mercodia, Uppsala, Sweden). This assay is based on a monoclonal antibody (4E6) directed against a conformational epitope in the apoB-100 moiety of LDL, which is generated as a consequence of reaction of lysine residues with aldehydes.[25] The intra-assay and inter-assay coefficients of variation were 4.8 and 7.8%, respectively.

Statistical analysis

Statistical analysis was performed with Stata 7 for Windows. Patients continuing trial participation after the baseline measurements were analyzed according to intention-to-treat principle. Outcome variables were analyzed with generalized estimating equations (GEE) with ADMA (primary) as dependent variable. The primary independent variable in the GEE model was treatment strategy (1= intervention group, 0=placebo group) adjusted for time and baseline observations with an independent working correlation structure. To assess the effect at the different time points, time was treated as a categorical variable and represented by dummy variables. Subsequently, effect modification by time was evaluated by adding the product-term of intervention and time (intervention X time) as independent variable to the model.

GEE assess the effect of the treatment steps on ADMA while adjusting for within subject's dependency associated with the repeated observations.[26] If the dependent variable was not normally distributed, analyses were performed after log-transformation. Additional analysis was performed by separating baseline values of plasma ADMA, homocysteine, and oxLDL into tertiles. A p-value less than 0.05 was considered as statistically significant.

Results

All subjects

Out of 93 included patients, 6 withdrew after the baseline measurement and 87 underwent at least one of the subsequent measurements (figure 1). In total, 36 subjects (77%) in the treatment group and 38 subjects (83%) in the placebo group completed the study. Four patients in the treatment group and two patients in the placebo group consumed between 60% and 80% of the study medication over the 24 months period; all others took at least 80% of their scheduled medication.

Baseline characteristics are reported in table 1. The mean ± SD ADMA and SDMA concentration in the total study population were 0.53 ± 0.07 μmol/L and 1.14 ± 0.46 μmol/L respectively (normal: 0.50 ± 0.06 μmol/L and 0.53 ± 0.10 μmol/L in a sample of healthy individuals aged 50-75 years).[27]

After adjustment for baseline ADMA values and time, the treatment strategy had no significant overall effect on plasma ADMA concentrations (ß= - 0.006; P= 0.27 for between-group difference after 24 months; figure 2) and additional adjustments for baseline renal function, oxLDL, CRP and homocysteine did not have any influence on this result. After the vitamin E intervention step (t=12 months), ADMA concentrations were significantly lower in the treatment group compared to placebo (ß= -0.022; P= 0.02 for between-group difference at 12 months, adjusted for baseline) (figure 2) and this effect remained significant also after additional adjustments for baseline renal function, oxLDL, CRP and homocysteine (data not shown).

Stratification according to baseline plasma ADMA, homocysteine and oxLDL concentration

Results of the stratified analyses in the predefined strata are presented in figure 3. In tertiles of baseline ADMA and oxLDL concentrations, the treatment strategy did not show an effect on ADMA concentrations (P=0.96 and P= 0.90 respectively for statistical interaction). There was no effect-modification by homocysteine concentration (P=0.60) but when subjects were stratified in tertiles of baseline homocysteine concentrations, ADMA concentrations significantly decreased in the stratum with the highest baseline homocysteine concentrations (>24.3 μmol/L) relative to placebo (ß= -0.028; P= 0.03 for between-group difference after 24 months, adjusted for baseline ADMA). Additional adjustment for oxLDL, CRP concentrations and renal function during the study did not change this effect (ß= -0.026; P= 0.04 for between-group difference after 24 months). The most marked reduction in ADMA in this group occurred after the addition of B-vitamins (i.e. between 12 and 24 months; ß= -0.044; P=0.06 for between-group difference at 24 months, adjusted for baseline ADMA).

Effect of the treatment strategy on plasma SDMA concentration

After adjustment for baseline SDMA values and time, the treatment strategy had no significant effect on SDMA (ß=0.006; P= 0.78 for between group difference after 24 months: Figure 4). No significant between-group difference was found at the different time points and in the subgroup analyses according to baseline ADMA, oxLDL and homocysteine concentration (data not shown).

Discussion

In this study we found no overall effect of a multi-step anti-oxidant treatment strategy consisting of pravastatin, vitamin E and B vitamins on ADMA in stage 3 and 4 CKD patients. A secondary analysis of individual treatment effects suggests that vitamin E treatment lowered ADMA concentrations significantly, but this effect disappeared after B-vitamin treatment was added. B-vitamin treatment reduced plasma ADMA concentration in subjects in the highest tertile of baseline homocysteine concentration.

Vitamin E as potential ADMA lowering agent

In our study , 6 months (between 6 to 12 months of therapy) of vitamin E treatment on top of pravastatin, led to a significant reduction in mean plasma ADMA concentrations in the intervention group compared to placebo. Only a handful of studies have been carried out so far to examine the effect of vitamin E (á-tocopherol) on ADMA in patients with renal disease. One study reported a significant decrease of ADMA in CKD patients after treat-

ment with vitamin E.[16] In a cross-over study in haemodialysis patients, a significant decrease in ADMA concentration after 6 months was observed in patients using a vitamin E-coated dialysis membrane.[28] In subjects with elevated traditional cardiovascular risk factors (without kidney disease), no effect of vitamin E on plasma ADMA concentration was found.[13]
The mechanism behind a possible effect of vitamin E is unclear. In CKD patients, increased oxidative stress[29] may increase the synthesis of ADMA by stimulating S-adenosylmethionine-dependent methyltransferases and/or by decreasing the breakdown of ADMA by reducing DDAH activity.[30] In addition, antioxidants have been shown to decrease ADMA concentration in cultured human endothelial cells and in-vivo in rats.[9;31] Despite the negative results with vitamin E in large clinical trials in patients with increased CVD risk, studies such as the SPACE trial, which showed a reduction in mortality in dialysis patients after vitamin E treatment[32] suggest a possible benefit in disease states where oxidative stress is increased. The extent to which such a beneficial effect could be explained by ADMA-lowering requires further studies. After addition of B-vitamins to the intervention, the difference in ADMA-levels between the treatment and the placebo group was no longer significant. Therefore, the isolated vitamin E effect should be interpreted with caution. Clearly, vitamin E does deserve further investigation as a potential ADMA- lowering agent, particularly in the context of CKD.

ADMA-lowering effects of B-vitamins

ADMA-lowering by B-vitamins was not reported in CKD before, but our results are in line with the results of a previous study which also showed a reduction in ADMA concentration after folate treatment in subjects with hyperhomocysteinemia.[12] Other studies found no effect of B-vitamins treatment on ADMA in normohomocysteinemic subjects with- or at risk for- cardiovascular disease.[13;15;33] Folic acid and vitamin B12 reduce homocysteine concentration by stimulating remethylation into methionine. Vitamin B6 stimulates the breakdown of homocysteine into cysteine via the transsulphuration pathway. Methionine is converted to S-adenosylmethionine (SAM) which serves as a methyl donor for protein arginine methyltransferases whereby ADMA is produced from proteolysis of the methylated arginine residues (figure 4). The demethylated product of SAM, S-adenosylhomocysteine (SAH), is hydrolyzed to homocysteine.[34] An elevated homocysteine concentration may increase the ADMA concentration by inhibition of the enzyme DDAH (figure 4).[8;10;35;36] B-vitamins lower plasma homocysteine concentrations[37] and could thus lower ADMA concentration. In our study we found no effect of the treatment on the concentrations of SDMA, another methylated product of L-arginine which is not metabolized by DDAH but excreted by the kidney.[1] This supports our hypothesis that increased activity of DDAH may be responsible for this reduction of plasma ADMA concentration in subjects with elevated homocysteine levels. As with the vitamin E effect, caution is also required for interpretation of the treatment effect in hyperhomocysteinemic patients, as this was a subgroup analysis, restricted to only 30 patients.

Statins and oxLDL in relation to ADMA

In the first six months of therapy, we found no effect of pravastatin on ADMA. One very recent study also demonstrated no effect of simvastatin on plasma ADMA concentration in CKD[19]. Also after stratification into tertiles of baseline oxLDL concentrations, we did not find

an effect of the intervention in subgroups. We previously reported a significant reduction in oxLDL and thereby a probable reduction in oxidative stress in our intervention group compared to the placebo group.[20] We thus expected a reduction in ADMA levels in the intervention group because reduced oxidative stress has been shown to reduce DDAH inhibition and thereby decrease levels of ADMA..

However, there is no gold standard for the detection of oxidative stress in vivo. Oxidized LDL concentrations are also influenced by the basal LDL-cholesterol levels and are not a reliable marker of oxidative stress. Finally, a recent study also showed no effect of oxLDL lowering on ADMA concentration.[13] In addition, we have also reported that our treatment strategy had no effect on plasma malondialdehyde levels[20]. Malondialdehyde is the most studied product of polyunsaturated fatty acid peroxidation. However, the specificity of commonly used tests (on the basis of derivatization with thiobarbituric acid [TBA]) to determine malondialdehyde is low as these tests also measure several compounds other than malondialdehyde which react with TBA.[38] F2-isoprostanes have recently gained recognition as reliable markers of oxidative stress [39]. However, we did not measure F2-isoprostanes in our population.

Symmetrical dimethylarginine and cardiovascular disease

We did not find any effect of the treatment strategy on SDMA concentration (figure 4). SDMA is not metabolised by DDAH but eliminated solely by renal excretion [40]. Fliser *et al*. recently demonstrated a very strong association between estimated glomerular filtration rate (eGFR) and SDMA[41]. In our primary analysis we could not demonstrate any effect of the treatment strategy on the eGFR in the treatment arm [20]. Therefore, we did not anticipate an effect of the treatment strategy on plasma SDMA.

The pathophysiological significance of SDMA is not fully elucidated. Previously, SDMA was thought to be biologically inactive[3]. A recent study demonstrated that SDMA might be a useful parameter for detecting patients in early stages of chronic kidney disease and for determining their risk for developing cardiovascular disease [42]. Recent data further suggest that SDMA may inhibit NO synthesis indirectly by limiting arginine availability to NO synthase[43].

ADMA lowering, endothelial function and common carotid intima-media thickness

One of the aims of our study was also to examine whether reduction in plasma ADMA with this treatment strategy could (partly) explain the reduction in common carotid IMT, endothelial function and microalbuminuria which were demonstrated in our primary analysis[20]. As the treatment strategy did not reduce in plasma ADMA we conclude that ADMA lowering was not responsible for these beneficial effects. Alternative mechanisms, however, are numerous, and include direct effects, alone or combined, of lipid lowering, oxidative stress reduction, and reduction of homocysteine.

Study limitations

Because of the multiple, additive interventions we were not able to assess the effect of the different treatment steps separately. Furthermore, we must point out that this study is a secondary analysis of an already published study and the study population is relatively small. Although 90 patients in our population had an estimated GFR of less than 60 ml/min/1.73 m^2, the levels of plasma ADMA in our population was relatively low and only 34% of

our population had plasma ADMA levels above the upper limit of the normal range of 0.54μmol/L. One can argue that this low mean ADMA levels may be one of the main reason for the negative outcome in our study. However, the treatment strategy also had no significant effect on plasma ADMA levels in 31 patients with a plasma ADMA level of above 0.54 μmol/L (figure 3). Therefore, in our opinion, the low mean ADMA levels in our population may not be a major reason for the negative results in this study. The sample size calculation in the ATIC study was originally based on assessing differences in common carotid artery intima-media thickness.[20] With a power of 80% and an alpha of 0.05, the smallest detectable difference in ADMA concentration that would be significant in the total study group is 0.045 μmol/L (i.e.: 0.75*SD). However, the small sample size may have influenced our results.

Conclusion

In conclusion, in the present study we found no overall effect of a multi-step anti-oxidant treatment strategy consisting of pravastatin, vitamin E and B vitamins on ADMA in stage 2-4 CKD patients. Possible ADMA-lowering effects of vitamin E deserve further attention, as do patients with very high homocysteine levels.

	Intervention (mean ± SD) n = 46	Placebo (mean ± SD) n = 47
Male gender,,n (%)	24 (51)	29 (63)
Age (years)	54 ± 11	52 ± 13
BMI (kg/m^2)	27 ± 5	26 ± 4
Smoking, n (%)	15 (32%)	17 (37%)
Antihypertensive medication n (%)		
ACE inhibitors	28 (85%)	33 (78%)
Angiotensin receptor blockers	10 (22%)	9 (19%)
Diuretics	18 (39%)	27 (57%)
ß-blockers	19 (41%)	15 (32%)
α-blockers	3 (6%)	2 (4%)
Calcium channel blockers	8 (17%)	13 (28%)
Plasma homocysteine (µmol/L)	20.2 ± 6.8	22.5 ± 11.3
OxLDL (U/L)	68 ± 12	61 ± 16
Plasma ADMA (µmol/L)	0.53 ± 0.06	0.53 ± 0.09
Plasma SDMA (µmol/L)	1.15 ± 0.45	1.11 ± 0.48
C-reactive protein (mg/L)	5.54 ± 6.59	5.53 ± 5.99
Renal function: MDRD Study equation, mL/s per m^2	0.53 ± 0.23	0.57 ± 0.23

Table 1. Baseline characteristics of the intervention and placebo group

Abbreviations: BMI , body mass index (weight in kilograms devided by height in meters squared); ACE, angiotensin-converting enzyme; oxLDL, oxidized low-density lipoprotein; ADMA, asymmetric dimethylarginine ; SDMA, symmetric dimethylarginine; MDRD, Modification of Diet in Renal Disease.
Conventional unit conversion factors: to convert MDRD equation to milliliters per minute per 1.73 m^2 , divide by 0.0167

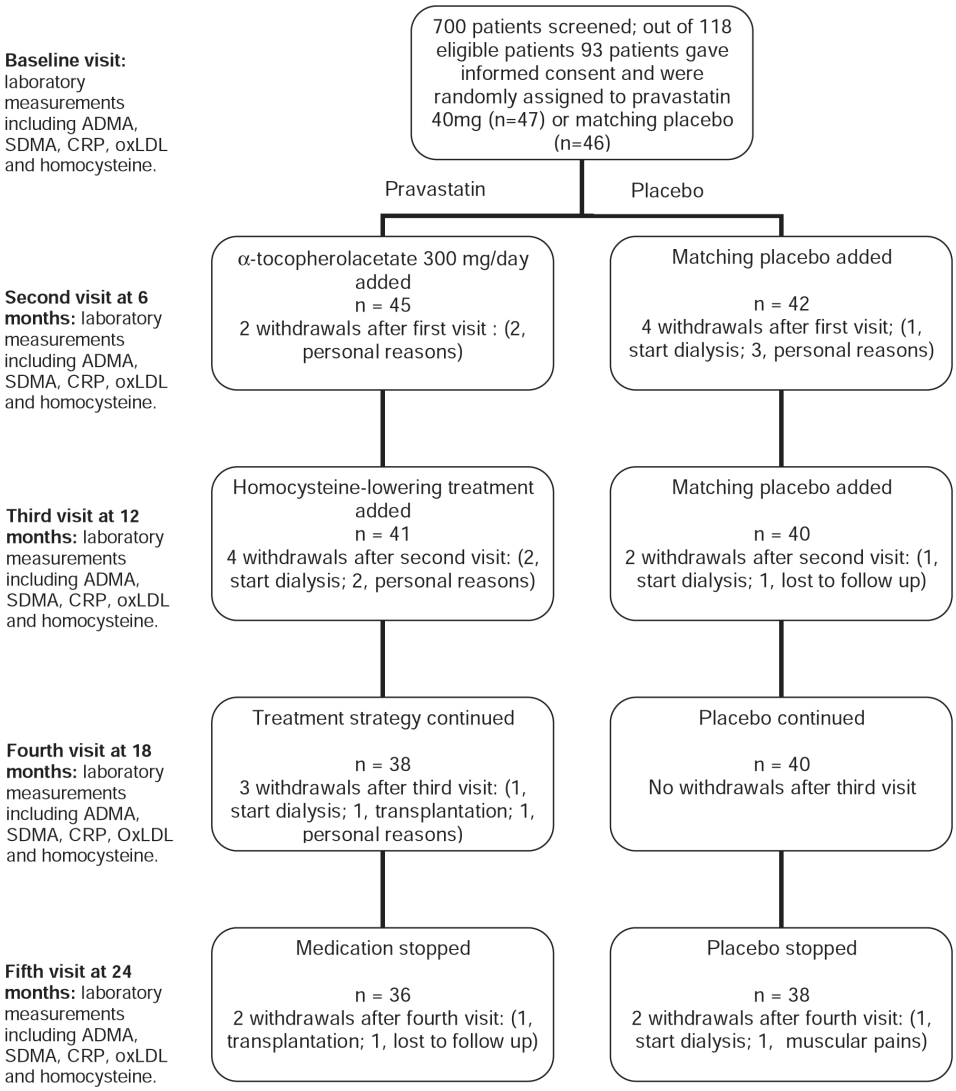

Figure 1: Flow of the participants through each stage of the study.
Abbreviations: ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; CRP, C-reactive protein: oxLDL, oxidized low-density lipoprotein.

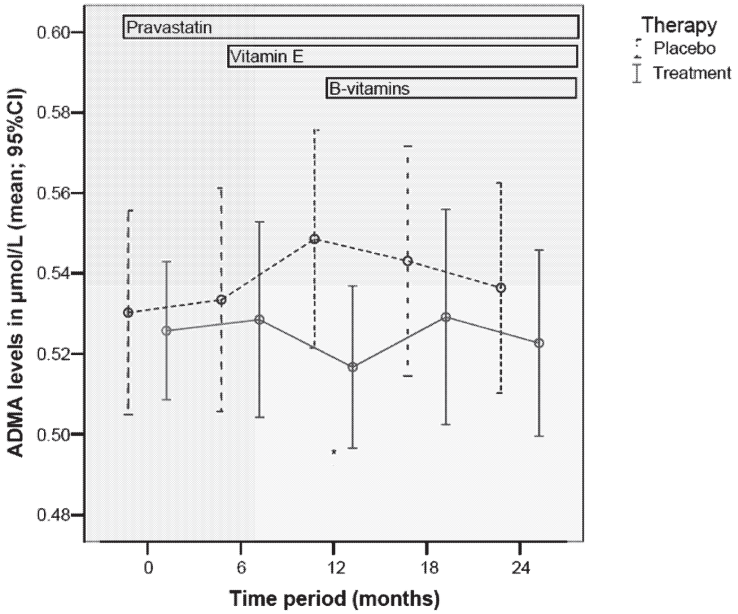

Figure 2: Mean and 95% confidence intervals of plasma concentrations of ADMA (µmol/L) following treatment with placebo; or with pravastatin, vitamin E and B-vitamins (N=93). Solid line refers to the intervention group following treatment with pravastatin, vitamin E and B-vitamins. Dotted line refers to the placebo group. *Difference between treatment and placebo; P=0.02.
(ADMA: asymmetric dimethylarginine; 95% CI: 95% confidence intervals for mean).

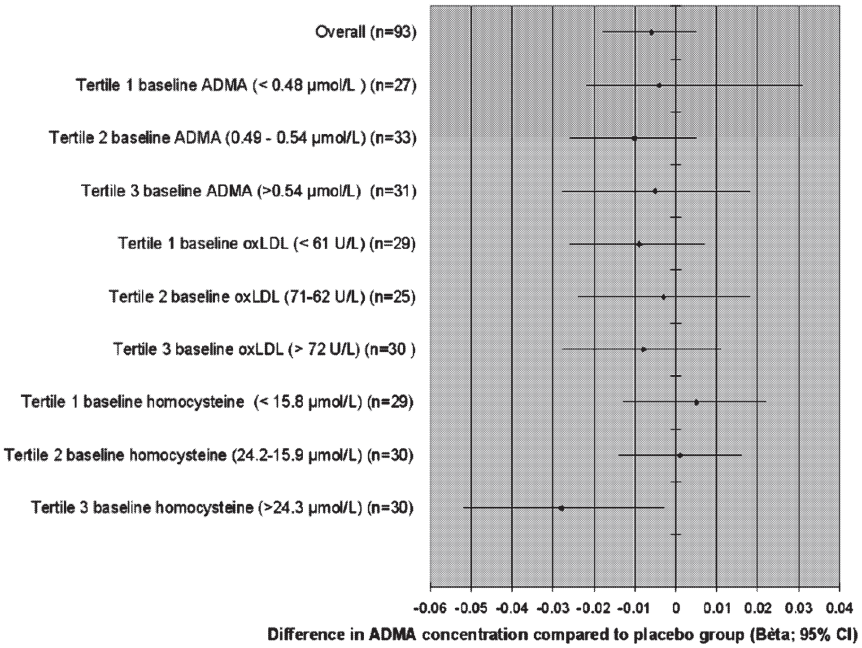

Figure 3: Between-group difference in plasma ADMA concentration in subgroup analysis after adjustment for baseline values and time without interaction time by treatment.
To convert homocysteine to milligrams per liter, divide by 7.397.
(ADMA: asymmetric dimethylerarginine; oxLDL: oxidized LDL; 95% CI: 95% confidence intervals for Bèta).

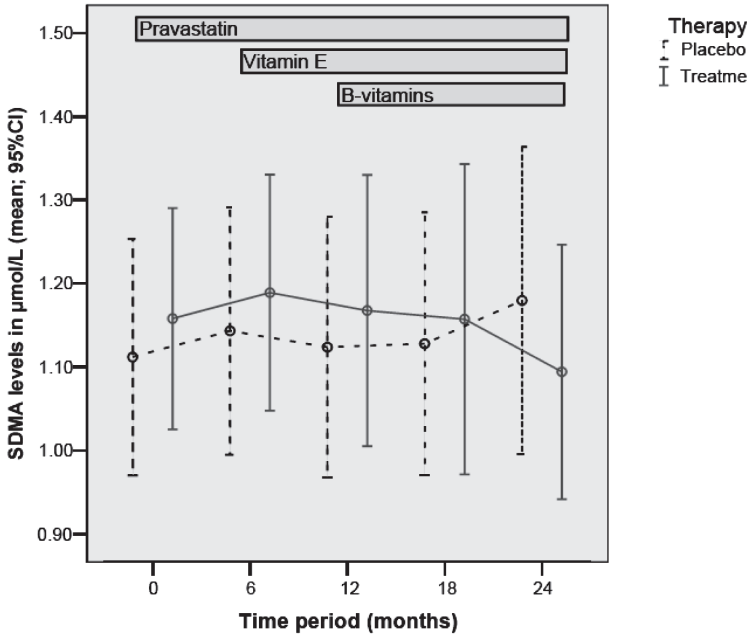

Figure 4: Mean and 95% confidence intervals of plasma concentrations of SDMA (µmol/L) following treatment with placebo; or with pravastatin, vitamin E and B-vitamins (N=93). Solid line refers to the intervention group following treatment with pravastatin, vitamin E and B-vitamins. Dotted line refers to the placebo group.
(SDMA: symmetric dimethylarginine; 95% CI: 95% confidence intervals for mean).

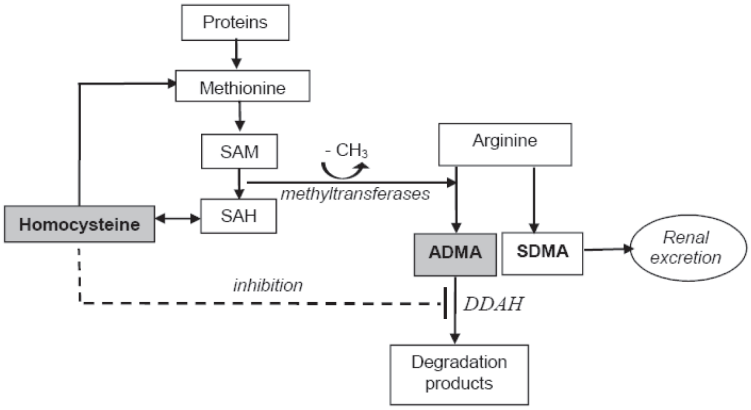

Figure 5: Homocysteine and ADMA pathway. (SAM; S-adenosylmethionine, SAH; S-adenosylhomocysteine, ADMA; asymmetric dimethylarginine, DDAH; dimethylarginine dimethylaminohydrolase. SDMA; symmetric dimethylarginine).

Reference List

1. Vallance P, Leiper J: Cardiovascular biology of the asymmetric dimethylarginine:dimethylarginine dimethylaminohydrolase pathway. Arterioscler Thromb Vasc Biol 24:1023-1030, 2004

2. Vallance P, Leone A, Calver A et al: Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 339:572-575, 1992

3. Zoccali C, Bode-Boger S, Mallamaci F et al: Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet 358:2113-2117, 2001

4. Furuki K, Adachi H, Matsuoka H et al: Plasma levels of asymmetric dimethylarginine (ADMA) are related to intima-media thickness of the carotid artery: an epidemiological study. Atherosclerosis 191:206-210, 2007

5. Miyazaki H, Matsuoka H, Cooke JP et al: Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. Circulation 99:1141-1146, 1999

6. Nanayakkara PW, Teerlink T, Stehouwer CD et al: Plasma asymmetric dimethylarginine (ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) concentration in patients with mild-to-moderate renal failure. Kidney Int 68:2230-2236, 2005

7. Zoccali C, Benedetto FA, Maas R et al: Asymmetric dimethylarginine, C-reactive protein, and carotid intima-media thickness in end-stage renal disease. J Am Soc Nephrol 13:490-496, 2002

8. Dayal S, Lentz SR: ADMA and hyperhomocysteinemia. Vasc Med 10 Suppl 1:S27-S33, 2005

9. Ito A, Tsao PS, Adimoolam S et al: Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase. Circulation 99:3092-3095, 1999

10. Stuhlinger MC, Oka RK, Graf EE et al: Endothelial dysfunction induced by hyperhomocyst(e)inemia: role of asymmetric dimethylarginine. Circulation 108:933-938, 2003

11. Piroddi M, Depunzio I, Calabrese V et al: Oxidatively-modified and glycated proteins as candidate pro-inflammatory toxins in uremia and dialysis patients. Amino Acids 32:573-592, 2007

12. Holven KB, Haugstad TS, Holm T et al: Folic acid treatment reduces elevated plasma levels of asymmetric dimethylarginine in hyperhomocysteinaemic subjects. Br J Nutr 89:359-363, 2003

13. Schmitt B, Wolters M, Kressel G et al: Effects of combined supplementation with B vitamins and antioxidants on plasma levels of asymmetric dimethylarginine (ADMA) in subjects with elevated risk for cardiovascular disease. Atherosclerosis 193:168-176, 2007

14. Spoelstra-de Man AM, Teerlink T, Brouwer CB et al: No effect of B vitamins on ADMA levels in patients at increased cardiovascular risk. Clin Endocrinol (Oxf) 64:495-501, 2006

15. Ziegler S, Mittermayer F, Plank C et al: Homocyst(e)ine-lowering therapy does not affect plasma asymmetrical dimethylarginine concentrations in patients with peripheral artery disease. J Clin Endocrinol Metab 90:2175-2178, 2005

16. Saran R, Novak JE, Desai A et al: Impact of vitamin E on plasma asymmetric dimethylarginine (ADMA) in chronic kidney disease (CKD): a pilot study. Nephrol Dial Transplant 18:2415-20, 2003

17. Lu TM, Ding YA, Leu HB et al: Effect of rosuvastatin on plasma levels of asymmetric dimethylarginine in patients with hypercholesterolemia. Am J Cardiol 94:157-161, 2004

18. Valkonen VP, Laakso J, Paiva H et al: Asymmetrical dimethylarginine (ADMA) and risk of acute coronary events. Does statin treatment influence plasma ADMA levels? Atheroscler Suppl 4:19-22, 2003

19. Panichi V, Mantuano E, Paoletti S et al: Effect of simvastatin on plasma asymmetric dimethylarginine concentration in patients with chronic kidney disease. J Nephrol 21:38-44, 2008

20. Nanayakkara PW, van Guldener C, ter Wee PM et al: Effect of a treatment strategy consisting of pravastatin, vitamin E, and homocysteine lowering on carotid intima-media thickness, endothelial function, and renal function in patients with mild to moderate chronic kidney disease: results from the Anti-Oxidant Therapy in Chronic Renal Insufficiency (ATIC) Study. Arch Intern Med 167:1262-1270, 2007

21. Cockcroft DW, Gault MH: Prediction of creatinine clearance from serum creatinine. Nephron 16:31-41, 1976

22. de Jong S, Teerlink T: Analysis of asymmetric dimethylarginine in plasma by HPLC using a monolithic column. Anal Biochem 353:287-289, 2006

23. Shipchandler MT, Moore EG: Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyzer. Clin Chem 41:991-994, 1995

24. Levey AS, Bosch JP, Lewis JB et al: A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med 130:461-470, 1999

25. Holvoet P, Vanhaecke J, Janssens S et al: Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. Circulation 98:1487-1494, 1998

26. Twisk JW: Longitudinal data analysis. A comparison between generalized estimating equations and random coefficient analysis. Eur J Epidemiol 19:769-776, 2004

27. Teerlink T: HPLC analysis of ADMA and other methylated L-arginine analogs in biological fluids. J Chromatogr B Analyt Technol Biomed Life Sci 851:21-29, 2007

28. Morimoto H, Nakao K, Fukuoka K et al: Long-term use of vitamin E-coated polysulfone membrane reduces oxidative stress markers in haemodialysis patients. Nephrol Dial Transplant 20:2775-2782, 2005

29. Locatelli F, Canaud B, Eckardt KU et al: Oxidative stress in end-stage renal disease: an emerging threat to patient outcome. Nephrol Dial Transplant 18:1272-1280, 2003

30. Boger RH, Sydow K, Borlak J et al: LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells: involvement of S-adenosylmethionine-dependent methyltransferases. Circ Res 87:99-105, 2000

31. Jiang JL, Li Ns NS, Li YJ et al: Probucol preserves endothelial function by reduction of the endogenous nitric oxide synthase inhibitor level. Br J Pharmacol 135:1175-1182, 2002

32. Boaz M, Smetana S, Weinstein T et al: Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. Lancet 356:1213-1218, 2000

33. Sydow K, Schwedhelm E, Arakawa N et al: ADMA and oxidative stress are responsible for endothelial dysfunction in hyperhomocyst(e)inemia: effects of L-arginine and B vitamins. Cardiovasc Res 57:244-252, 2003

34. Stam F, van Guldener C, ter Wee PM et al: Homocysteine clearance and methylation flux rates in health and end-stage renal disease: association with S-adenosylhomocysteine. Am J Physiol Renal Physiol 287:F215-F223, 2004

35. Herrmann W, Obeid R: Hyperhomocysteinemia and response of methionine cycle intermediates to vitamin treatment in renal patients. Clin Chem Lab Med 43:1039-1047, 2005

36. Lentz SR, Rodionov RN, Dayal S: Hyperhomocysteinemia, endothelial dysfunction, and cardiovascular risk: the potential role of ADMA. Atheroscler Suppl 4:61-65, 2003

37. Lobo A, Naso A, Arheart K et al: Reduction of homocysteine levels in coronary artery disease by low-dose folic acid combined with vitamins B6 and B12. Am J Cardiol 83:821-825, 1999

38. Del RD, Stewart AJ, Pellegrini N: A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis 15:316-328, 2005

39. Comporti M, Signorini C, Arezzini B et al: F2-isoprostanes are not just markers of oxidative stress. Free Radic Biol Med 44:247-256, 2008

40. Kielstein JT, Boger RH, Bode-Boger SM et al: Marked increase of asymmetric dimethylarginine in patients with incipient primary chronic renal disease. J Am Soc Nephrol 13:170-176, 2002

41. Fliser D, Kronenberg F, Kielstein JT et al: Asymmetric dimethylarginine and progression of chronic kidney disease: the mild to moderate kidney disease study. J Am Soc Nephrol 16:2456-2461, 2005

42. Bode-Boger SM, Scalera F, Kielstein JT et al: Symmetrical dimethylarginine: a new combined parameter for renal function and extent of coronary artery disease. J Am Soc Nephrol 17:1128-1134, 2006

43. Closs EI, Basha FZ, Habermeier A et al: Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B. Nitric Oxide 1:65-73, 1997

Chapter 8

Summary and concluding remarks

Patients with chronic kidney disease (CKD) have an unacceptably high risk of premature death, primarily as a result of cardiovascular disease (CVD) [1]. Several large, randomised trials in end stage renal disease (ESRD) patients have consistently shown no survival benefit from multiple new treatment strategies aimed at reducing CV disease. Earlier stages of CKD are common and are also associated with an increased risk of developing CVD. Traditional cardiovascular risk factors such as, age, dyslipidaemia, hypertension, diabetes mellitus, smoking and sedentary lifestyle, do not fully explain the cardiovascular mortality in these patients. The so-called novel risk factors such as increased oxidative stress, asymmetric dimethylarginine (ADMA), plasma adipokines, plasma homocysteine and DNA-hypomethylation are prevalent and thought to play an important role in development of atherosclerosis in CKD patients. However, the exact role of these factors in the pathogenesis of CV disease in CKD patients has not yet been elucidated. In addition, only a few cardiovascular intervention studies have been done in patients with mild to moderate CKD, whereas most of the large intervention trials with statins have excluded patients with moderate renal failure. Therefore, many important questions remained unanswered in this unfortunate patient group.

Anti-oxidant Therapy In chronic renal insufficiency (ATIC) study

With the aim of answering at least some of the above mentioned questions, in 2001 we started the Anti-oxidant Therapy In Chronic renal insufficiency (ATIC) study, a randomised, double-blind, placebo-controlled trial. We randomised 93 non-diabetic patients with mild to moderate chronic kidney disease (creatinine clearance 15-70 ml/min per 1.73 m^2 determined by Cockcroft-Gault formula), who were free of manifest atherosclerotic arterial disease, to receive a regimen that included pravastatin 40mg/day followed by á tocopherol acetate (vitamin E) 300mg/day and homocystiene lowering therapy (folic acid 5mg/day, pyridoxine hydrochloride 100mg/day, cyanocobalamin 1 mg/day) or matching placebo tablets for each consecutive six months period. The primary aim of the study was to evaluate the effect of this stepwise treatment strategy on common carotid artery intima-media thickness (CCA-IMT) and brachial artery flow-mediated vasodilatation (BA-FMD), both strong surrogate markers of cardiovascular disease. We further decided to evaluate the effects of this strategy on albuminuria and estimated glomerular filtration rate (eGFR). We also planned to use the baseline data of this study to evaluate the associations between renal function and several non-traditional risk factors such as asymmetric dimethylarginine (ADMA), plasma adipokines (plasma adiponectin and plasma leptin), and global leukocyte DNA hypomethylation. We also intended to evaluate the associations between these novel risk factors and CCA-IMT and thereby to get some insight into the possible pathophysiological role these factors play in excess cardiovascular risk in CKD patients. In addition, we decided to assess the effect of this treatment strategy on plasma ADMA levels and global leukocyte DNA-methylation and to study whether eventual reduction in these risk factors/markers could explain the beneficial effects (if any) of this therapeutic strategy on the measured surrogate endpoints.

Why oxidative stress lowering and what was the rationale behind this peculiar design?

During the design of the study we postulated, on the basis of a few published studies, that the high cardiovascular mortality rates in ESRD patients could be partly explained by enhanced oxidative stress [2-4] and that the oxidative stress hypothesis should be considered

as a unifying concept of increased cardiovascular disease in CKD patients [5]. It was already known that increased oxidative stress occurs in early stages of CKD [6] and oxidation of low-density lipoprotein (LDL) is thought to be a key step in the initiation of the early atherosclerotic lesion [7]. Therefore, we decided to look for interventions which would create oxidative stress reduction in our population.
In 1996, Stephens *et al* published the results of Cambridge Heart Antioxidant (CHAOS) study in which a population of patients with angiographically proven coronary atherosclerosis was treated with alpha-tocopherol 800 IU in a randomised controlled manner. The treatment significantly reduced the risk of developing the primary endpoint of cardiovascular death and non-fatal myocardial infarction [8]. In addition, although most of the interventions aimed at reducing cardiovascular disease in ESRD patients yielded disappointing results, the SPACE-study, in which 196 patients with ESRD who were treated with 800 IU vitamin E in a randomised controlled fashion, demonstrated encouraging results on composite cardiovascular endpoints and myocardial infarction [9]. It was also shown that plasma homocysteine was strongly related to the renal function and that virtually all ESRD patients had elevated homocysteine levels which also predicted cardiovascular outcomes in these patients [10]. It was further postulated that the vasculotoxic effects of homocysteine were caused by increased oxidative stress [11]. In addition, studies in various populations demonstrated that statins decreased cardiovascular endpoints spectacularly [12;13].
After considering all these facts thoroughly, we decided to use a strategy which consisted of pravastatin which was thought to reduce the substrate LDL-cholesterol and thereby reduce oxidized LDL, vitamin E which acts as a free-radical scavenger to reduce free oxygen radicals and a combination of B-vitamins to reduce homocysteine, because high homocysteine was thought to increase oxidative stress. We decided to use these treatment modalities on top of each other on the one hand to reduce the number of people needed to perform the study and on the other hand to achieve a maximum oxidative stress reduction (benefit) in the treatment arm and thereby increase the power of the study. Because we thought that the maximum effect of each treatment would be achieved in six months, we decided to add each new intervention every six months. In addition, even at that time it was known that there was an unequivocal relationship between hypertension and progression of CKD, and that inhibition of the renin-angiotensin system with ACE-inhibitors would lead to a significant reduction in decline in eGFR and cardiovascular complications [14]. Therefore, we decided to control the blood pressure in both the treatment and control arm aggressively (<140/90mmHg) and all the subjects who were not on ACE-inhibitors or ARBs were put on ACE-inhibitors at inclusion. Although currently lower blood pressure targets are recommended, especially in patients with proteinuria > 1g/day, during the design of the study many guidelines recommended 140/90 mmHg as the target blood pressure, and we decided to adhere to this target strictly during the study period.

What have we learned from the ATIC study?
In chapter 3 of this thesis we demonstrate, for the first time, that plasma adiponectin had a significant, non-linear, inverse association with eGFR and that in the multivariate analysis eGFR had the strongest correlation with plasma adiponectin. In addition, adiponectin had no influence on the significant association between estimated glomerular filtration rate (eGFR) and a surrogate marker of endothelial dysfunction (plasma von Willebrand factor) and a marker of leukocyte-endothelial cell adhesion [soluble vascular leukocyte cell adhe-

sion molecule-1 (sVCAM-1)]. We, therefore, postulated that the increased adiponectin levels in CKD patients are primarily a reflection of impaired kidney function. Although renal function strongly predicted plasma leptin in our population, body mass index and insulin resistance also had a strong association with plasma leptin in univariate and multivariate analysis. Plasma leptin also did not explain the known associations between kidney function and endothelial dysfunction and leukocyte-endothelial cell adhesion. Future studies are needed to clarify the role of leptin in CKD patients.

Adipose tissue has a physiological role beyond mere storage of fat and recent interest has focused on the role of adipokines, such as adiponectin and leptin, both as protectors and promoters of vascular disease in CKD [15;16]. However, the pathophysiological role of adiponectin in excess CVD in CKD patients is not clear, as published studies have shown contradicting results [17]. For example, while Guebre-Egziabher *et al.* reported that the increase in adiponectin in patients with CKD is explained primarily by patient's body composition and the altered metabolic parameters[18], Mitsnefes *et al.* attributed this increase primarily to the decline in kidney function[19]. While one study reported that lower adiponectin levels are associated with increased risk of cardiovascular events in CKD patients, [20] others reported that high rather than low adiponectin levels predicts mortality in both CKD and congestive heart failure[21]. In addition, the functional role of increased leptin in CKD patients is also unclear[22]. We planned to investigate the association between renal function and plasma leptin and adiponectin in a population with a wide range of eGFR so that we could shed some light on these matters. Therefore, we pooled the baseline data from two studies which were being performed in the same centres as the ATIC study. Our data show that eGFR was the main determinant of plasma adiponectin and also that plasma adiponectin and leptin did not explain the known associations between eGFR and endothelial dysfunction. The limitations of the study design are described in detail in chapter 3.

In chapter 4, using baseline data from the ATIC-study, we demonstrated that, in patients with mild to moderate renal failure, eGFR was inversely associated with plasma ADMA level. We also demonstrated, for the first time, that plasma ADMA was in turn associated with CCA-IMT and plasma soluble vascular cell adhesion molecule-1. We conclude that plasma ADMA may be one of the mechanisms that link mild to moderate renal failure with cardiovascular disease. During the design of the ATIC study it was already known that plasma ADMA concentrations were high and were strongly and independently related to CCA-IMT in patients with ESRD [23]. However, data on the association between plasma ADMA and CCA-IMT were not available at that moment, and there were conflicting data on the association between eGFR and plasma ADMA in patients with mild to moderate kidney failure[24;25]. Thus our findings were novel and thought provoking.

In chapter 5 we demonstrate that in patients with mild to moderate CKD, the global DNA-methylation was not associated with renal function or with CCA-IMT or BA-FMD. In addition we also showed that the above-mentioned treatment strategy had no influence on DNA- methylation in this population. Therefore, we concluded that DNA-hypomethylation probably has no significant role in the pathogenesis of cardiovascular disease in patients with CKD. During the design of the study, hyperhomocysteinaemia was strongly implicated in the development and progression of atherothrombotic vascular disease [26]. The pathophysiological explanation for this link was initially thought to be a direct vasculotoxic effect of homocysteine itself [27]. However, an alternative view was that hyperhomocysteinaemia

itself is not harmful but indirectly inhibits methyl fluxes during transmethylation of methionine and thereby leads to a decreased methylation of DNA. Global DNA-hypomethylation was thought to be associated with various diseases including atherosclerotic vascular disease[28]. Global DNA-hypomethylation has been demonstrated in ESRD patients [29] and was implicated as an important candidate contributing to CVD in these patients [30]. However, it was thought that leukocyte DNA-hypomethylation in ESRD patients may be caused by leukocyte activation on dialysis membranes and data on patients with mild to moderate renal failure and DNA-hypomethylation were inconclusive [31]. As mentioned earlier, results of many studies to examine the effects of therapeutic homocysteine lowering has been disappointing. We, unfortunately, also could not demonstrate an association between DNA-hypomethylation and eGFR and our treatment strategy also did not alter global DNA-methylation. Our results suggest that the role of global DNA-hypomethylation as a risk factor for CVD in patients with CKD, if any, is limited.

In chapter 6 we demonstrated that 18 months of the stepwise treatment strategy, on top of well-controlled blood pressure, achieved a significant reduction in CCA-IMT and significant increase in BA-FMD in the active treatment arm after adjustments for baseline values. Increased urinary albumin excretion was also attenuated by the treatment strategy although there were no observed beneficial effects on renal function (eGFR). Unfortunately, the individual effects of each intervention could not be determined and also the study was not powered to detect a clinically important difference in hard cardiovascular endpoints. We concluded that in patients with mild to moderate chronic kidney disease, 18 months of an oxidative stress lowering treatment strategy along with well-controlled blood pressure reduced CCA-IMT and urinary albumin excretion and increased BA-FMD. In addition, the treatment strategy, especially pravastatin, was associated with a low rate of adverse events.

In chapter 7 we demonstrate that the above-mentioned treatment strategy had no significant effect on plasma ADMA levels. However, analysis of separate treatment effects suggested that vitamin E significantly lowered ADMA in the treatment group compared to the placebo group. This effect disappeared after addition of homocysteine lowering treatment to the treatment arm. We concluded that our treatment strategy had no effect on plasma ADMA levels, and hence the observed reduction in CCA-IMT and improvement BA-FMD in our population could not be explained by a change in ADMA levels. In the next paragraph, we describe in detail the implications of our findings.

What were the drawbacks in the ATIC study?

The most accurate way to investigate the determinants of the excess cardiovascular risk in patients with CKD, compared to patients without CKD, is to perform a prospective population-based cohort study with exclusion of patients with cardiovascular disease at the baseline. Although the ATIC study was a prospective study we designed this study primarily to investigate the treatment effects. Although we have published three papers describing cross sectional associations using baseline data of our study, these findings do not permit any final conclusions with regard to the causality of the described associations. In addition, we decided to include patients using ACE-inhibitors or angiotensin receptor blockers (ARB) and patients not using these agents were put on ACE-inhibitors before the initiation of the study. Oxidative stress can also be reduced by ACE-inhibitors and ARBs [32]. ACE-inhibitors

also lower plasma ADMA [33;34]. This may have influenced all our cross sectional analyses and may also have reduced the power of our study. However, since the use of ACE-inhibitors in CKD, especially in the presence of any level of proteinuria, was considered standard treatment even during the design of our study, it made sense to evaluate our anti-oxidant strategy on top of treatment with ACE-inhibitors. Although diabetes mellitus is expected to be one of the major causes of CKD in the future we excluded patients with diabetes mellitus. By excluding patients with diabetes as well as patients with previous CVD, we expected to recruit a population with increased oxidative stress primarily caused by their kidney failure. We, therefore, studied a selected population of patients with mild-to-moderate CKD and this study unfortunately had limited power and was of too short duration to detect an effect on clinical cardiovascular endpoints.

In the last few years results of large, randomised, controlled trials with vitamin E and homocysteine lowering have not shown any beneficial effects on cardiovascular events in different populations [35-38]. However, during the period we designed our study the available information at that time suggested, these vitamins to have beneficial effects in patients with renal failure because these patients were known to have increased oxidative stress and small studies with vitamin E in dialysis patients at that time showed some promising results [39]. At the same time, many studies showed an undeniable link between homocysteine , renal function and cardiovascular disease, and during that time homocysteine lowering was thought to be one of the best potential interventions to reduce CV disease in CKD patients [10;39]. We decided to use the treatment strategies on top of each other with six monthly intervals and planned to evaluate the effects of individual treatments separately. We expected (in retrospect, wrongly) the maximum effect of each intervention to be achieved within six months after the given intervention, and/or that the additional effect of the next step would be clearly distinguishable from the effects of the previous step. Lowering of CCA-IMT and improvement in BA-FMD were observed during the whole study period (chapter 6). In retrospect, we were unable to draw any conclusions on individual effects of these interventions. The treatment modalities of the present study certainly have effects independent of oxidative stress lowering. In addition, there is no gold standard for the detection of oxidative stress in vivo. We measured the oxidized LDL concentration which is unfortunately also influenced by the basal LDL-cholesterol levels. We measured total malondialdehyde (MDA) levels in plasma, i.e. the most studied product of polyunsaturated fatty acid peroxidation. However, the specificity of commonly used tests to determine MDA is low as these tests also measure several compounds other than MDA[40]. F2-isoprostanes have recently gained recognition as reliable markers of oxidative stress[41]. At the moment we are in the process of introducing the measurement of F2-isoprostanes in our laboratory and we hope to measure the F2-isoprostanes in our study population in the near future. Until then, we cannot draw any conclusions whether the observed improvements were the results of oxidative stress lowering or other effects such as reduction of lipid levels.

Where should we go from here?

While it is widely known that patients with ESRD are at greatly increased risk of developing CV disease, it is not well known among the general physicians, that patients with only mild to moderate kidney disease are also at increased risk. The clear association between slightly reduced kidney function and cardiovascular risk may, at least partly, be the result of a relationship between total atherosclerotic burden and decreased renal function, because

intrarenal atherosclerosis (ischaemic renal disease) is a common cause of reduced renal function in patients with atherosclerosis. However, there are clear indications that impaired eGFR is an independent risk factor for developing cardiovascular disease in the general population [42]. In spite of this, not a single risk calculator, which are recommended for physicians, uses eGFR to calculate the risk of developing CV disease. This is puzzling. In our opinion, most patients with mild kidney disease are not diagnosed and most physicians do not measure creatinine clearance during routine check ups. Measurement of only plasma creatinine can underestimate the renal function especially in old and thin patients. Thus mild to moderate kidney failure is, in our opinion, underdiagnosed. Several guidelines have been formulated, both nationally and internationally, to assist the physicians to diagnose and treat patients with kidney disease. However it is well known that patients do not reach treatment goals formulated in these guidelines [43]. This also applies to patients with kidney disease [44;45]. Thus, apart from being underdiagnosed, CKD is also not treated properly. Furthermore, most of these patients come to nephrologists when they reach stage 4 which is in my opinion already too late for cardiovascular preventive measures. In patients with diabetes mellitus and heart failure a multifactorial intervention significantly improved metabolic control and reduced cardiovascular events significantly[46;47] Therefore, much benefit may be achieved effectively with a multifactorial approach addressing risk factors such as blood pressure, serum lipids, dietary meausres, physical exercise et cetera in mild to moderate CKD. The MASTERPLAN study, a randomised, controlled trial examining the effect of such a multifactorial approach is already underway in The Netherlands and hopefully this study will shed more light on this matter in the future. In the mean time, general physicians and practitioners should be urged to consider mild to moderate CKD a cardiovascular risk factor and perhaps routine creatinine measurement should automatically generate estimated GFR which would make it easy for physicians to diagnose CKD.

The ATIC study is one of the first clinical trials primarily designed to examine the effects of a treatment strategy which included pravastatin on surrogate markers of CV disease in CKD patients. We did demonstrate strong favourable effects on these markers with our treatment strategy and, in my opinion pravastatin certainly played a key role in these effects. Also very few subjects stopped the therapy because of side-effects. However, long term studies with clinical endpoints are needed to confirm these results and some are already underway. One of the largest is the SHARP study, a randomised, double blind, placebo-controlled trial which investigates the effects of cholesterol reduction with simvastatin and ezetimibe in around 6000 patients with moderate CKD (plasma creatinine > 150 μmol/L in men and 130 μmol/L in women) and 3000 patients with ESRD on major vascular events; the estimated study completion date is July 2010 (ClinicalTrials.gov Identifier: NCT00125593). In addition, the Heart Outcomes Prevention Evaluation-3 (HOPE-3 study), which examines the effects of combined blood pressure lowering and lipid lowering with rosuvastatin 10mg a day, is underway at this moment, and this study will also include patients with CKD (ClinicalTrials.gov Identifier: NCT00468923). Unfortunately studies with homocysteine lowering in various populations including CKD patients have given disappointing results [48].
The SPACE study, which assessed the effect of vitamin E 800 iu/day versus placebo in 196 haemodialysis patients with preexisting CV disease over a median period of 519 days, demonstrated a significant reduction in cardiovascular endpoints. However, the Heart Out-

comes Prevention Evaluation study (HOPE study), which included 993 subjects with serum creatinine concentration between 125 and 200 μmol/L, found no effect on cardiovascular outcomes of treatment with vitamin E 400 iu/day compared to placebo. The apparent disparity in findings between SPACE and HOPE may be explained by the higher dose of vitamin E used, the greater severity of kidney disease and the three fold higher CV event rate in the SPACE trial. Many guideline groups have therefore considered the SPACE trial preliminary and demanded further studies before recommending vitamin E therapy routinely for CKD patients. A large study with antioxidant combination vitamin E and alpha lipoic acid in stage 3 and 4 CKD patients is underway (ClinicalTrials.gov Identifier: NCT00308971).

There is growing evidence to support the hypothesis that high plasma ADMA, an endogenous inhibitor of NO synthase, is associated with increased risk of CVD [49]. Although we could demonstrate a significant reduction in CCA-IMT and a significant increase in BA-FMD our treatment strategy had no effect on plasma ADMA. Therefore, reduction in ADMA could not explain these favourable effects. This is on the one hand disappointing and on the other hand encouraging. The question remains whether pharmacological reduction in ADMA would lead to more favourable effects on these vascular parameters. Small studies have demonstrated that drugs such as fenofibrate[50;51], thiazolidinediones[52], ACE-inhibitors and ARBs[34;53], vitamin E and even rosuvastatin[54]can reduce ADMA[55]. We did demonstrate a significant reduction in ADMA during treatment with vitamin E which disappeared after addition of homocysteine lowering therapy. However, large scale studies to examine the effects of ADMA reduction on CV events are, to our knowledge, not underway. In our opinion, long term studies with vitamin E or other agents to examine the effect of possible ADMA reduction on vascular endpoints are of great interest.

Recent studies show that the adipose tissue is a complex organ with functions far beyond the storage of fat, and secretes adipokines such as adiponectin and leptin[56]. Evidence suggests that these signaling molecules are linked to insulin resistance, systemic inflammation and uraemic anorexia in patients with CKD[57;58]. As discussed in detail in chapter 3, the exact pathophysiological role of these adipokines in CKD is far from clear. Further research is needed to investigate the complex interactions between adipokines signaling networks and their effects on vascular health and outcome in CKD.

In addition, there are exciting new fields such as the role of fibroblast growth factor 23 (FGF 23) and vitamin D deficiency in chronic kidney disease[59]. Several factors including parathyroid hormone (PTH) and vitamin D play a critical role in maintaining plasma phosphate levels [60]. FGF 23 has recently been identified as an important regulator of systemic phosphate balance[61]. Under physiological conditions FGF23 promotes phosphaturia and suppresses the 1 Ü-hydroxylase activity, thus leading to a reduction in 1,25-dihydroxyvitamin D levels. The phosphate balance is altered in CKD and in these patients very high levels of FGF23 have been demonstrated[62]. As the number of viable nephrons decreases in CKD, in spite of the high FGF23 the net phosphate excretion does not increase sufficiently. This high phosphate level in combination with the reduction in 1,25-dihydroxyvitamin D levels leads to secondary hyperparathyroidism. A strong association has been described between hyperphosphataemia, hyperparathyroidism and CV disease in CKD[63]. This increased CV disease is probably caused by increased vascular calcification and evidence is emerging that optimizing treatment of calcium and phosphate alterations may decrease CV risk in

CKD patients[64]. However, the exact role of these new factors such as FGF23 in patients with CKD has not yet been elucidated and is in our opinion an exciting challenge for the future.

The terms risk factor reversal, paradoxical risk factors or reverse epidemiology refer to alterations in the normal relation between risk factors and clinical outcomes. In particular populations this abnormal relation can be so severe that this can result in more or less the exact opposite or reversal of the usual association between a risk factor and clinical outcome that is found in the general population. Such risk factor reversal is commonly observed in patients with advanced CKD[65]. This phenomenon of reverse epidemiology makes it sometimes difficult to target traditional risk factors in an effective manner because determination of an optimal target for risk factors such as blood pressure and LDL-cholesterol has become uncertain, especially in patients with advanced stages of CKD. Therefore, randomised controlled trials will certainly be necessary to ascertain the optimal levels for these risk factors in CKD and ESRD patients.
In spite of a long and hard journey by many research groups the puzzle of increased cardiovascular disease in CKD has certainly not yet been resolved. The ATIC study has left us with more questions than answers. The journey is, in our opinion, far from over.

Reference List

1. Foley RN, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998; 32: S112-S119

2. Loughrey CM, Young IS, Lightbody JH, McMaster D, McNamee PT, Trimble ER. Oxidative stress in haemodialysis. *QJM* 1994; 87: 679-683

3. Toborek M, Wasik T, Drozdz M, Klin M, Magner-Wrobel K, Kopieczna-Grzebieniak E. Effect of hemodialysis on lipid peroxidation and antioxidant system in patients with chronic renal failure. *Metabolism* 1992; 41: 1229-1232

4. Zima T, Haragsim L, Stipek S, Bartova V, Nemecek K. Lipid peroxidation on dialysis membranes. *Biochem Mol Biol Int* 1993; 29: 531-537

5. Himmelfarb J, Stenvinkel P, Ikizler TA, Hakim RM. The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney Int* 2002; 62: 1524-1538

6. Oberg BP, McMenamin E, Lucas FL *et al*. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 2004; 65: 1009-1016

7. Parthasarathy S, Santanam N, Auge N. Oxidized low-density lipoprotein, a two-faced Janus in coronary artery disease? *Biochem Pharmacol* 1998; 56: 279-284

8. Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996; 347: 781-786

9. Boaz M, Smetana S, Weinstein T *et al*. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *Lancet* 2000; 356: 1213-1218

10. Mallamaci F, Zoccali C, Tripepi G *et al*. Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. *Kidney Int* 2002; 61: 609-614

11. Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR, Haynes WG. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. *Circulation* 1999; 100: 1161-1168

12. Shepherd J, Cobbe SM, Ford I *et al*. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995; 333: 1301-1307

13. Sacks FM, Pfeffer MA, Moye LA *et al*. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 1996; 335: 1001-1009

14. Brenner BM. Retarding the progression of renal disease. *Kidney Int* 2003; 64: 370-378

15. Zoccali C, Mallamaci F, Tripepi G *et al*. Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease. *J Am Soc Nephrol* 2002; 13: 134-141

16. Scholze A, Rattensperger D, Zidek W, Tepel M. Low serum leptin predicts mortality in patients with chronic kidney disease stage 5. *Obesity (Silver Spring)* 2007; 15: 1617-1622

17. Mitsnefes M, Kartal J, Khoury P, Daniels S. Adiponectin in children with chronic kidney disease: role of adiposity and kidney dysfunction. *Clin J Am Soc Nephrol* 2007; 2: 46-50

18. Guebre-Egziabher F, Bernhard J, Funahashi T, Hadj-Aissa A, Fouque D. Adiponectin in chronic kidney disease is related more to metabolic disturbances than to decline in renal function. *Nephrol Dial Transplant* 2005; 20: 129-134

19. Mitsnefes M, Kartal J, Khoury P, Daniels S. Adiponectin in children with chronic kidney disease: role of adiposity and kidney dysfunction. *Clin J Am Soc Nephrol* 2007; 2: 46-50

20. Zoccali C, Mallamaci F, Tripepi G *et al*. Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease. *J Am Soc Nephrol* 2002; 13: 134-141

21. Menon V, Li L, Wang X *et al*. Adiponectin and mortality in patients with chronic kidney disease. *J Am Soc Nephrol* 2006; 17: 2599-2606

22. Axelsson J, Stenvinkel P. Role of fat mass and adipokines in chronic kidney disease. *Curr Opin Nephrol Hypertens* 2008; 17: 25-31

23. Zoccali C, Benedetto FA, Maas R *et al*. Asymmetric dimethylarginine, C-reactive protein, and carotid intima-media thickness in end-stage renal disease. *J Am Soc Nephrol* 2002; 13: 490-496

24. Wahbi N, Dalton RN, Turner C, Denton M, Abbs I, Swaminathan R. Dimethylarginines in chronic renal failure. *J Clin Pathol* 2001; 54: 470-473

25. Kielstein JT, Boger RH, Bode-Boger SM *et al*. Marked increase of asymmetric dimethylarginine in patients with incipient primary chronic renal disease. *J Am Soc Nephrol* 2002; 13: 170-176

26. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA* 2002; 288: 2015-2022

27. van Guldener C, Stehouwer CD. Hyperhomocysteinaemia and vascular disease—a role for DNA hypomethylation? *Lancet* 2003; 361: 1668-1669

28. Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. *Biochim Biophys Acta* 2007; 1775: 138-162

29. Ingrosso D, Cimmino A, Perna AF *et al*. Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. *Lancet* 2003; 361: 1693-1699

30. Perna AF, Capasso R, Lombardi C, Acanfora F, Satta E, Ingrosso D. Hyperhomocysteinemia and macromolecule modifications in uremic patients. *Clin Chem Lab Med* 2005; 43: 1032-1038

31. Stenvinkel P, Karimi M, Johansson S *et al*. Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease? *J Intern Med* 2007; 261: 488-499

32. Schleicher E, Friess U. Oxidative stress, AGE, and atherosclerosis. *Kidney Int Suppl* 2007; S17-S26

33. Ito A, Egashira K, Narishige T, Muramatsu K, Takeshita A. Angiotensin-converting enzyme activity is involved in the mechanism of increased endogenous nitric oxide synthase inhibitor in patients with type 2 diabetes mellitus. *Circ J* 2002; 66: 811-815

34. Chen JW, Hsu NW, Wu TC, Lin SJ, Chang MS. Long-term angiotensin-converting enzyme inhibition reduces plasma asymmetric dimethylarginine and improves endothelial nitric oxide bioavailability and coronary microvascular function in patients with syndrome X. *Am J Cardiol* 2002; 90: 974-982

35. Alkhenizan AH, Al-Omran MA. The role of vitamin E in the prevention of coronary events and stroke. Meta-analysis of randomized controlled trials. *Saudi Med J* 2004; 25: 1808-1814

36. Bleys J, Miller ER, III, Pastor-Barriuso R, Appel LJ, Guallar E. Vitamin-mineral supplementation and the progression of atherosclerosis: a meta-analysis of randomized controlled trials. *Am J Clin Nutr* 2006; 84: 880-887

37. Albert CM, Cook NR, Gaziano JM *et al*. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. *JAMA* 2008; 299: 2027-2036

38. Jamison RL, Hartigan P, Kaufman JS *et al*. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. *JAMA* 2007; 298: 1163-1170

39. Boaz M, Smetana S, Weinstein T *et al*. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *Lancet* 2000; 356: 1213-1218

40. Del RD, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 2005; 15: 316-328

41. Comporti M, Signorini C, Arezzini B, Vecchio D, Monaco B, Gardi C. F2-isoprostanes are not just markers of oxidative stress. *Free Radic Biol Med* 2008; 44: 247-256

42. Vanholder R, Massy Z, Argiles A, Spasovski G, Verbeke F, Lameire N. Chronic kidney disease as cause of cardiovascular morbidity and mortality. *Nephrol Dial Transplant* 2005; 20: 1048-1056

43. Clinical reality of coronary prevention guidelines: a comparison of EUROASPIRE I and II in nine countries. EUROASPIRE I and II Group. European Action on Secondary Prevention by Intervention to Reduce Events. *Lancet* 2001; 357: 995-1001

44. Tonelli M, Bohm C, Pandeya S, Gill J, Levin A, Kiberd BA. Cardiac risk factors and the use of cardioprotective medications in patients with chronic renal insufficiency. *Am J Kidney Dis* 2001; 37: 484-489

45. Wheeler DC, Townend JN, Landray MJ. Cardiovascular risk factors in predialysis patients: baseline data from the Chronic Renal Impairment in Birmingham (CRIB) study. *Kidney Int Suppl* 2003; S201-S203

46. Gaede P, Vedel P, Larsen N, Jensen GV, Parving HH, Pedersen O. Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. *N Engl J Med* 2003; 348: 383-393

47. Vale MJ, Jelinek MV, Best JD *et al*. Coaching patients On Achieving Cardiovascular Health (COACH): a multicenter randomized trial in patients with coronary heart disease. *Arch Intern Med* 2003; 163: 2775-2783

48. Jamison RL, Hartigan P, Kaufman JS *et al*. Effect of homocysteine lowering on mortality and vascular disease in advanced chronic kidney disease and end-stage renal disease: a randomized controlled trial. *JAMA* 2007; 298: 1163-1170

49. Kielstein JT, Zoccali C. Asymmetric dimethylarginine: a cardiovascular risk factor and a uremic toxin coming of age? *Am J Kidney Dis* 2005; 46: 186-202

50. Yang TL, Chen MF, Xia X, Luo BL, Li YJ. Effect of fenofibrate on the level of asymmetric dimethylarginine in individuals with hypertriglyceridemia. *Eur J Clin Pharmacol* 2006; 62: 179-184

51. Yang TL, Chen MF, Luo BL, Xie QY, Jiang JL, Li YJ. Fenofibrate decreases asymmetric dimethylarginine level in cultured endothelial cells by inhibiting NF-kappaB activity. *Naunyn Schmiedebergs Arch Pharmacol* 2005; 371: 401-407

52. Wang TD, Chen WJ, Cheng WC, Lin JW, Chen MF, Lee YT. Relation of improvement in endothelium-dependent flow-mediated vasodilation after rosiglitazone to changes in asymmetric dimethylarginine, endothelin-1, and C-reactive protein in nondiabetic patients with the metabolic syndrome. *Am J Cardiol* 2006; 98: 1057-1062

53. Scalera F, Martens-Lobenhoffer J, Bukowska A, Lendeckel U, Tager M, Bode-Boger SM. Effect of telmisartan on nitric oxide—asymmetrical dimethylarginine system: role of angiotensin II type 1 receptor gamma and peroxisome proliferator activated receptor gamma signaling during endothelial aging. *Hypertension* 2008; 51: 696-703

54. Lu TM, Ding YA, Leu HB, Yin WH, Sheu WH, Chu KM. Effect of rosuvastatin on plasma levels of asymmetric dimethylarginine in patients with hypercholesterolemia. *Am J Cardiol* 2004; 94: 157-161

55. Beltowski J, Kedra A. Asymmetric dimethylarginine (ADMA) as a target for pharmacotherapy. *Pharmacol Rep* 2006; 58: 159-178

56. Axelsson J, Stenvinkel P. Role of fat mass and adipokines in chronic kidney disease. *Curr Opin Nephrol Hypertens* 2008; 17: 25-31

57. Guebre-Egziabher F, Drai J, Fouque D. Adiponectin and chronic kidney disease. *J Ren Nutr* 2007; 17: 9-12

58. Axelsson J. Obesity in chronic kidney disease: good or bad? *Blood Purif* 2008; 26: 23-29

59. Gal-Moscovici A, Sprague SM. Role of vitamin D deficiency in chronic kidney disease. *J Bone Miner Res* 2007; 22 Suppl 2: V91-V94

60. Fukagawa M, Kazama JJ. With or without the kidney: the role of FGF23 in CKD. *Nephrol Dial Transplant* 2005; 20: 1295-1298

61. Schiavi SC, Kumar R. The phosphatonin pathway: new insights in phosphate homeostasis. *Kidney Int* 2004; 65: 1-14

62. Shimada T, Hasegawa H, Yamazaki Y *et al*. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *J Bone Miner Res* 2004; 19: 429-435

63. Zoccali C. Traditional and emerging cardiovascular and renal risk factors: an epidemiologic perspective. *Kidney Int* 2006; 70: 26-33

64. Ritz E. The clinical management of hyperphosphatemia. *J Nephrol* 2005; 18: 221-228

65. Kalantar-Zadeh K, Block G, Humphreys MH, Kopple JD. Reverse epidemiology of cardiovascular risk factors in maintenance dialysis patients. *Kidney Int* 2003; 63: 793-808

Chapter 9

Nederlandse samenvatting en dankwoord

Nederlandse samenvatting

Hart- en vaatziekten zijn de belangrijkste doodsoorzaak van dialysepatiënten en dit beperkt hun levensverwachting sterk. Er zijn steeds meer aanwijzingen dat milde tot matige nierschade een onafhankelijke risicofactor is voor hart- en vaatziekten. Bekende (traditionele) cardiovasculaire risicofactoren, zoals hoge bloeddruk, hypercholesterolemie (hoog cholesterolgehalte), roken en diabetes mellitus (suikerziekte), verklaren deze oversterfte bij nierpatiënten niet volledig. Er lijken dus andere factoren aanwezig te zijn die een rol spelen bij de ontwikkeling van hart- en vaatziekten. In dit proefschrift worden onderzoeken beschreven waarin de invloed van enkele niet-traditionele risicofactoren op het verhoogde risico op hart- en vaatziekten bij nierpatiënten nader is bestudeerd. Veder onderzochten wij de invloed van een meervoudige behandelingsstrategie bestaande uit verlaging van cholesterol, beperking van zuurstofschade (oxidatieve stress) en optimalisatie van B-vitamine-inname op vroege tekenen van vaatschade.

Niet-traditionele risicofactoren

Oxidatieve stress:

Vrije zuurstofradicalen zijn bijproducten van de normale stofwisseling en kunnen cellulaire processen verstoren en schade aan cellen veroorzaken. Toename van zuurstofradicalen, al dan niet gepaard gaande met met afname van antioxidanten (stoffen die zuurstofradicalen neutraliseren) wordt ‘oxidatieve stress’ genoemd. Oxidatie van bepaalde cholesterolhoudende deeltjes in het bloed (low density lipoproteïne (LDL cholesterol) lijkt een belangrijke rol te spelen in de initiatie van atherosclerose, het proces dat leidt tot vaatvernauwing en vaatafsluiting. Er zijn steeds meer aanwijzingen dat oxidatieve stress verhoogd is bij patiënten met nierinsufficiëntie en bij hen een belangrijke rol speelt bij verhoogde risico op hart- en vaatziekten.

Adipokines:

Er zijn steeds meer argumenten dat vetweefsel een ‘endocrine functie’ heeft. Dat wil zeggen dat het weefsel stoffen produceert die elders in het lichaam biologische effecten teweegbrengen. Vetweefsel produceert verschillende stoffen die adipokines worden genoemd. Tot de adipokines behoren de hormonen leptine en adiponectine. Adipokines zouden een belangrijke rol kunnen spelen in de pathofysiologie van ziekten die geassocieerd zijn met obesitas, zoals (neiging tot) suikerziekte en atherosclerose. Aan leptine worden bijvoorbeeld eigenschappen toegedicht die atherosclerose zouden kunnen bevorderen. Adiponectine daarentegen heeft ontstekingsremmende en atherosclerose-remmende eigenschappen. Tegen de verwachting in is de bloedconcentratie van adiponectine verhoogd in patiënten met nierinsufficiëntie. Deze toename wordt slechts gedeeltelijk verklaard door achteruitgang van de nierfunctie. De bloedconcentratie van leptine is verhoogd bij patiënten met nierinsufficiëntie en leptine lijkt een rol te spelen in dialyse-geassocieerde vermagering. De mogelijke functionele rol die deze hormonen spelen in de verhoogde atherogeniciteit in patiënten met nierinsufficientie is op dit moment onduidelijk.

Homocysteine en DNA-hypomethylering:

Nierfunctieverlies gaat gepaard met verhoogde bloedconcentraties van het eiwit homocysteine (een product van stofwisseling van het aminozuur methionine) en van het

hieraan gerelateerde eiwit S-adenosylhomocysteine (SAH). Bekend is dat verhoogde SAH de DNA-methylering {koppeling van methylgroepen (drie waterstofmoleculen gekoppeld aan een koolstofmolecuul) aan het DNA-molecule} kan remmen. Bij dialyse patiënten is in eerder onderzoek verlaagde methylering van DNA (hypomethlering) aangetoond. DNA-hypomethylering is mogelijk geassocieerd met een verhoogde kans op hart en vaatziekten. Of er een associatie is tussen mild tot matig nierfunctie verlies en DNA-hypomethylering is onbekend.

Asymmetrisch Dimethylarginine (ADMA):
Stijging van een van de natuurlijke afbraakproducten van eiwiten, ADMA (Asymmetrisch Dimethylarginine) is mogelijk mede verantwoordelijk voor het toegenomen risico op hart- en vaatziekten bij dialysepatiënten. ADMA is een remmer van NO (nitric oxide; stikstofmonoxide) en veroorzaakt daardoor een verstoorde werking van de cellen van de binnenbekleding van de bloedvaten, het endotheel. De rol van verhoogd ADMA in het ontstaan van hart- en vaatziekten bij milde tot matig nierinsufficiëntie is onduidelijk.

Surrogaateindpunten van cardiovasculaire ziekten bij patiënten met chronische nierinsufficiëntie
De dikte van de slagaderwand wordt vooral bepaald door de mate van aderverkalking (atherosclerose). Uit meerdere studies is gebleken dat er een verband bestaat tussen de vaatwanddikte (intima-media-dikte) in de halsslagader en de mate van aderverkalking op andere plaatsen in het lichaam. Dit verband is zowel in de algemene populatie als in patiënten met nierinsufficiëntie aangetoond. Veel van de bovengenoemde risicofactoren veroorzaken een verstoorde werking van de cellen van de binnenbekleding van de bloedvaten (endotheeldisfunctie). Adequate functie van het endotheel kan onder andere gemeten worden door endotheel-afhankelijke vaatverwijding van arteria brachialis (de slagader naar de onderarm), gemeten met ultrageluidreflecties (ultrasonografie). Er zijn duidelijk aanwijzingen dat endotheeldisfunctie samenhangt met hoge kans op ontwikkeling van hart- en vaatziekten.

Opbouw van het proefschrift:

Anti-oxidant Therapy In Chronic renal insufficiency (ATIC) studie:
Wij hebben de laatste jaren een gerandomiseerd, placebo-gecontroleerd onderzoek verricht bij 93 patiënten met een matig tot ernstig gestoorde nierfunctie (gemeten aan een glomerulaire filtratiesnelheid van 15-70 ml/min per 1.73m^2; normaal is deze waarde rond de 100 ml/min per 1.73 m2) De actief behandelde groep werd behandeld met een cholesterolverlager (pravastatine 40mg). Na 6 maanden werd het anti-oxidatieve vitamine E (300mg/dag) toegevoegd. Na 12 maanden werd homocysteïneverlagende therapie bestaande uit foliumzuur (5mg/dag), vitamine B_{12} (1mg/dag) en vitamine B_6 (100mg/dag) toegevoegd. De placebo-groep kreeg op elk van deze tijdpunten een nepmedicijn toegediend. Tijdens het onderzoek werd gekeken naar de effecten op: [1] de intima-mediadikte van de halsslagader [2] de endotheelfunctie, door het meten van de endotheel-afhankelijke vaatverwijding in de onderarmslagader en door de bepaling van tekenen van endotheelschade in het bloed. Gedurende de studie werden op meerdere tijdstippen ook verschillende oxidatieve-stressparameters worden bepaald (ox-LDL, malondialdhyde), alsmede adiponectine, leptine, ADMA en methylering van DNA.

Onze onderzoeksvragen waren

1. Is er een associatie tussen nierfunctie en bovengenoemde niet-traditionele risico factoren zoals plasma adiponectine, plasma leptine, DNA-hypomethylering en plasma ADMA spiegels?
2. Zijn er associaties tussen plasma ADMA en DNA-hypomethylering met eerdergenoemde surrogaateindpunten, zoals intima-media dikte van halsslagader en endotheelfunctie?
3. Wat is de invloed van bovengenoemde behandelingsstrategie op twee sterke surrogaateindpunten voor hart- en vaatziekten: intima-media dikte van halsslagader en endotheelfunctie, gemeten door endotheel-afhankelijke vaatverwijding in de onderarmslagader

In hoofdstuk 2 worden de ingewikkelde associaties tussen homocysteine en ADMA en hun eventuele pathosfysiologische rol in het verhoogde risico op hart- en vaatziekten in nierpatiënten besproken. Er zijn sterke associaties beschreven tussen verhoogd homocysteine, verhoogd ADMA en hart- en vaatziekten. Maar de associatie tussen plasma ADMA en homocysteine bij nierpatiënten blijkt zwak te zijn. De sterke associatie die beschreven is tussen plasma ADMA enerzijds, en intima-media-dikte en hart- en vaatziekten anderzijds, is onafhankelijk van homocysteine. Schadelijk effecten van ADMA en homocysteine op de vaatwand worden dus waarschijnlijk via verschillende mechanismen veroorzaakt.

In hoofdstuk 3 hebben wij aangetoond dat de adiponectineconcentratie in het bloed stijgt bij een afnemende nierfunctie. Adiponectine blijkt echter geen invloed te hebben op het verband tussen nierfunctie en tekenen van endotheelschade (gemeten aan de bloedspiegel van von-Willebrandfactor). Ook de leptine spiegel bleek hoger bij patiënten met verminderde nierfunctie. In tegenstelling tot adiponectine werd deze stijging echter vooral verklaard door metabole veranderingen.

In hoofdstuk 4 hebben wij de data van de ATIC-studie gebruikt om aan te tonen dat nierfunctieverlies gepaard gaat met een stijging van de ADMA-concentratie. Verder hebben wij, voor de eerste keer, aangetoond dat een hoge ADMA-concentratie in het bloed gepaard gaat met een toegenomen intima-mediadikte van de halsslagader. We hebben daarom geconcludeerd dat ADMA mogelijk een belangrijke rol speelt bij de hoge kans op het ontwikkelen van hart- en vaatziekten bij patiënten met mild tot matig nierfalen

In hoofdstuk 5 hebben wij aangetoond dat globale DNA-methylering geen verband heeft met nierfunctie, intima-media dikte of endotheelfunctie. Verder hebben wij geen effect kunnen aantonen van de gegeven therapieën in de ATIC-studie op DNA-methylering. Wij concludeerden dat DNA-hypomethylering waarschijnlijk geen rol speelt in de hogere kans op hart- en vaatziekten bij patiënten met chronisch nierfalen.

In hoofdstuk 6 demonstreerden wij dat de 18 maanden durende behandeling die wij in de ATIC-studie toepasten een significante daling van de intima-media dikte van de halsslagader en een significante verbetering van de endotheelfunctie veroorzaakte. De behandeling had geen invloed op de achteruitgang van de nierfunctie. Helaas konden wij de afzon-

derlijke effecten van de drie therapieën niet los van elkaar bekijken, zodat onduidelijk blijft of één van de toegepaste behandelingen, of alleen de combinatie ervan tot dit gewenste resultaat leidt. Onze conclusie was dat combinatietherapie met pravastatine, vitamine E en homocysteineverlaging een significante verbetering in de toestand van de vaatwand gaf, en daarmee wellicht een gunstig effect op het risico op hart- en vaatziekten heeft.

In hoofdstuk 7 toonde wij aan dat terwijl de toegepaste behandeling in de ATIC-studie geen invloed had op ADMA, Vitamine E gaf mogelijk wel een afname van ADMA, maar dit effect was niet meer aanwezig nadat de homocysteine-verlagende therapie was toegevoegd. Wij concludeerden dat een afname van ADMA de gunstige effecten op de vaatwand in onze populatie niet kan verklaren.

In hoofdstuk 8 gaan wij uitgebreid in op het totstandkomen van de ATIC-studie en motiveren wij de keuze voor 3 achtereenvolgende therapiestappen . De nadelen van deze onderzoeksstrategie worden verder besproken. In de laatste alinea hebben wij gefilosofeerd over waar wij in de toekomst heen moeten in de zoektocht naar oorzaken en therapieën voor hart- en vaatziekten in patiënten met nierfalen. In onze optiek heeft de inspanning van vele mensen nog geen definitieve antwoorden gegeven op talloze vragen. De ATIC-studie heeft, zoals vaker het geval is bij onderzoek, vragen beantwoord, maar tegelijkertijd nog meer, nieuwe vragen gegenereerd. Er blijft nog veel werk over voor de toekomst.

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List of publications:

1. Thijs A, Nanayakkara PWB, ter Wee PM, Huijgens PC, van Guldener C, Stehouwer CDA. Mild-to-moderate renal impairment is associated with platelet activation: a cross-sectional study. *Clinical Nephrology*. In press

2. Kortmann W, van Agtmael MA, van Diessen J, Kanen BLJ, Nanayakkara PWB. 5-Oxoroline a s a cause of high anion gap metabolic acidosis: an uncommon cause with common risk factors. *Neth J Med*. 2008;66:354-357.

3. Nanayakkara PWB, Kiefte-de Jong JC, ter Wee PM, Stehouwer CDA, van Ittersum FJ, Olthof MR, Teerlink T, Twisk JWR, van Guldener C, Smulders Y. A treatment strategy consisting of pravastatin, vitamin E and homocysteine lowering does not lower plasma asymmetric dimethylarginine (ADMA) concentration in patients with mild to moderate kidney disease: a randomized, double-blind, placebo-controlled trial. *Am J Kidney Dis*. 2008 Sep 9. [Epub ahead of print]

4. del Sol AI, Nanayakkara PWB. Prvastatin: an evidence-based statin? *Expert Opin Drug Metabol Toxicol*. 2008;4:821-825.

5. Nanayakkara PWB, Kiefte- de Jong JC, Stehouwer CDA, van Ittersum FJ, Olthof MR, Kok RM, Blom HJ, van Guldener C, ter Wee PM, Smulders Y. Association between global leukocyte DNA methylation, renal function, carotid intima-media thickness and plasma homocysteine in patients with stage 2-4 chronic kidney disease. *Nephrol Dial Transplant*. 2008;23:2586-2592.

6. Nanayakkara PWB, van Guldener C, ter Wee PM, Scheffer PG, van Ittersum FJ, Twisk JWR, Teerlink T, van Dorp W, Stehouwer CDA. Effect of an oxidative-stress-reducing strategy consisting of pravastatin, vitamin E and homocysteine-lowering on carotid intima-media thickness, endothelial function and renal function in patients with mild-to-moderate chronic kidney disease. *Arch int Med.* 2007;167:1262-1270.

7. Nanayakkara PWB, van der Veldt AA, Simsek S, Smulders YM, Rauwerda JA. Verapamil-induced erytheralgia. *Neth J Med.* 2007;65:349-351.

8. Van Guldener C, Nanayakkara PWB, Stehouwer CDA. Homocysteine and asymmetric dimethylarginine (ADMA): biochemically linked but differently related to vascular disease in chronic kidney disease. *Clin Chem Lab Med*. 2007;45:1683-1687.

9. Nanayakkara PWB, van Bunderen CC, Santerse B. A bumpy ride to a discarded diagnosis. *Lancet*. 2007;369:436.

10. Nanayakkara PWB, van Bunderen CC, Simsek S, Smulders YM, Pals G. Ehlers-Danlos syndrome, the vascular type, in a patient with ruptured aneurysm of the splenic artery. *Neth J Med*. 2006;64:374-376.

11. Simsek S, de Vries XH, Jol JAD, Spoelstra-de Man AME, Nanayakkara PWB, Smulders YM, Mahieu HF, ter Wee PM. Sino-nasal bony and cartilaginous destruction associated with cocaine abuse, S. aureus and antinetrophil cytoplasmic antibodies. *Neth J Med.* 2006;64:248-251.

12. Volman MNM, Serne EH, Voskuyl AE, Nanayakkara PW. Het fenomeen van Raynaud en de rol van capillairmicroscopie. *Nederlands Tijdschrift voor Dermatologie en Venereologie.* 2005;15:326-328.

13. Nanayakkara PWB, Teerlink T, Stehouwer CDA, Allajar D, Spijkerman A, Schalkwijk C, ter Wee PM, van Guldener C. Plasma asymmetric dimethylarginine (ADMA) concentration is independently associated with carotid intima-media thickness and plasma soluble vascular adhesion molecule-1 (sVCAM) concentration in patients with mild-to-moderate renal failure. *Kidney Int.* 2005;68:2230-2236.

14. Nanayakkara PWB, Meijboom M, Schouten JA. Suicidal and aggressive thoughts as a result of taking a Hypericum preparation. *Ned Tijdschr Geneeskd.* 2005;149:1347-1349.

15. Hofstee HM, Nanayakkara PWB, Stehouwer CDA. Acute hepatitis related to prednisolone. *Eur J Intern Med..* 2005;16:209-210.

16. Nanayakkara PWB, de Jong E, Postmus PE. Bilateral pulmonary infiltrates in a patient with ulcerative colitis receiving mesalazine. *Eur J Intern Med*. 2005;16:209-210.

17. Simsek S, Van den Berg FG, Nanayakkara PWB, Rauwerda JA, Stehouwer CDA. Anomaly of the inferior vena cava causing recurrent deep vein thrombosis in a young male. *Eur J Intern Med..*2004;15:251-254.

18. Susanto CR, Nanayakkara PWB. The skin is the clue. *Eur J Intern Med.*2000;14:493-494.

19. Simsek s, Nanayakkara PWB. Hereditary hyperferritinaemia-cataract syndrome. *Ned Tijdschr Geneeskd.* 2003;147:2557.

20. Simsek S, Nanayakkara PWB, Keek JM, Faber LM, Bruin KF, Pals G. Two Dutch families with hereditary hyperferritinaemia-cataract syndrome. *Neth J Med.*2003;61:291-295.

21. Schram MT, Stam F, de Jongh RT, de Vries G, van Dijk RA, Serne EH, Lampe D, Nanayakkara PWB, Tushuizen ME, Scheffer PG, Schalkwijk CG, Kamper AM, Stehouiwer CDA. The effect of calcium debesilate on vascular endothelial function, blood pressure, and markers of oxidation in obese male smokers. *Atherosclerosis*. 2003;170:59-72.

22. Nanayakkara PWB, Hartdorff CM, Stehouwer CD, Vermeulen RJ, de Visser M. A man with fever and a persistent handgrip. *Lancet.* 2003;362:1038.

23. van Guldener C, Nanayakkara PW, Stehouwer CD. Homocysteine and blood pressure. *Curr Hypertens Rep.* 2003;5:26-31.

24. Nanayakkara PWB, van Doorn J, van den Berg FG, van Groeningen CJ, Pinedo HM, Hoekman K. Treatment of haemangiopericytoma-associated hypoglycemia with embolisation. *Eur J Intern Med.* 2002;13:340-343.

25. Zweegman S, Vermeer MH, Bekkink MW, van der Valk P, Nanayakkara P, Ossenkoppele GJ. Leukaemia cutis: clinical features and treatment strategies. *Haematologica*. 2002;87:ECR13.

26. Nanayakkara PWB, Gans ROB, Reichert-Thoen J, ter Wee PM. Serous retinal detachment as an early presentation of thrombotic thrombocytopenic purpura. *Eur J Intern Med.* 2000;11:286-288.

27. Nanayakkara PWB, le Poole CY, Fouque D , van Guldener C, Stehouwer CDA Smulders YM, van Ittersum FJ, Siegert CEH , Drai J , Kostense PJ, ter Wee PM. Plasma adiponectin concentration has an inverse and a non linear association with estimated glomerular filtration rate in patients with K/DOQI 3-5 chronic kidney disease. *Submitted*.

28. de Lange TE, Simsek S, Kramer MHH, Nanayakkara PWB. A case of Cocaine induced panhypopituitarism with Human Neutrophil Elastase specific Anti Neutrophil Cytoplasmic Antibodies. *Submitted.*

29. Thijs A, Nanayakkara PWB, van Asseldonk DP, ter Wee PM, Ittersum FJ, Huijgens P, van Guldener C, Stehouwer CDA. A treatment strategy consisting of pravastatin, vitamin E and homocysteine-lowering medication is associated with reduced blood platelet lysosomal degranulation in patients with mild-to-moderate renal impairment: Results from the Anti-oxidant Therapy In Chronic renal insufficiency study. *Submitted*

Curriculum Vitae:

Prabath Nanayakkara was born on 6th October 1963 in Wattala, Sri Lanka. He started his primary education in de Mazanod College Kandana. Upon receiving a government sponsored scholarship in 1974, he joined Ananda college Colombo, a premier Buddhist school in Sri Lanka. He successfully completed his advanced level examination in 1981 with exceptional results and was accepted into the medical faculty of the University of Colombo in 1982. He got through his second MBBS examination in June 1984 with a Second class (Upper Division) honours and a distinction in Physiology. In 1986, when all universities in Sri Lanka were closed indefinitely due to the civil unrest in the country, he formed a pop band and started pursuing a music carrier. In October 1989, he fled Sri Lanka as a result of the Civil unrest and ended up in Wagenberg, The Netherlands. A year later the Dutch government granted him a visa on compassionate grounds. In July 1990 he passed the Dutch proficiency examination of the Tilburg University (Katholieke Universiteit Brabant) and joined the medical faculty of the Vrije Universiteit Amsterdam in March 1991. He obtained his masters degree (doctoraal) in March 1992 and his MD in December 1994. In January 1995 he started his fellowship in internal medicine in the VU University medical centre in Amsterdam under the supervision of Prof. Dr. Jan van der Meer. Immediately after finishing his fellowship in January 2001 he joined the department of internal medicine as a consultant physician. Two years later he obtained his fellowship in vascular medicine. Currently he is attached to the VU University medical centre as a senior stafmember of the department of internal medicine and the Head of the outpatient internal medicine department.